

TIRC

Grants

1954-60

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Comm:

Drs. Lynch, Chm.  
Jacobson  
Reimann

TOBACCO INDUSTRY RESEARCH COMMITTEE

150 East Forty Second Street, New York 17, N.Y.

Application for Research Grant

Date: August 8, 1960

1. Name of Investigator: Hobart A. Reimann, M.D.
2. Title: Professor of Medicine
3. Institution & Address: Hahnemann Medical College and Hospital  
230 North Broad Street  
Philadelphia 2, Pa.
4. Project or Subject: The Effect of Tobacco or Nicotine in Periodic Disorders.
5. Detailed Plan of Procedure:

Periodic disease, a newly recognized syndrome, was brought to medical attention in 1948 by the applicant (reprint attached). More than 500 cases have since been reported from world-wide sources, comprised chiefly of periodic peritonitis but of periodic fever, arthralgia and neutropenia as well. They rarely are fatal in themselves, but the episodes repeated at short intervals for years are incapacitating, prevent steady employment and thus cause economic loss for the victims. Thus far, the cause is unknown. There are no known means of prevention or cure. Males are chiefly affected.

Of importance is an investigation of the immediate cause or inciting factors of episodes. All evidence points to a neural or neurovascular disturbance of central origin. This is supported by the work of Dunbar, of Wolff and others on the neurovascular cause of a variety of sterile inflammatory and other reactions.

Since nicotine first stimulates then depresses the sympathetic nervous system, and the vagus nerve, besides having other complicated central effects, tests should be made to see if the use of tobacco in any form or nicotine alone incites or modifies episodes through central neural action. Abdominal pain is the most distressing feature of periodic peritonitis, and tobacco smoking in excess in some persons (including the Director of the Project) causes painful esophageal and intestinal spasm. The question of allergy also was raised by several observers as a cause. Allergy to tobacco or its constituents may play a role in periodic disease.

Studies are planned to observe the effects of using tobacco or of injecting nicotine during episodes and in free intervals. Any clinical effects or reactions of tissues, capillaries, electrical resistance, dermal temperature, sweating and others would be observed. Results may lead to other approaches. Such studies would require hospitalization of some patients for 2 or more weeks to cover a cycle or two, and others can be observed and tested as out-patients.

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It is of importance also to inquire by personal query or by mailed questionnaire with as many victims of periodic diseases as possible to determine their habits regarding the use of tobacco and any effects therefrom on the nature of the episodes that they may have noted.

6. Budget Plan:

* Salaries:	Salaries	\$ 8,600.00 *
Director of Project: \$5,000	Expendable Supplies	500.00
(As part-time, that I will	Permanent Equipment	(none)
need from other clinical	Overhead (15%)	1,440.00
duties to supervise and	Other (Hospitalization, \$17/day)	500.00
engage in this investigation)	Total	\$11,040.00
Technician..... \$3,600;	(No charge for medical	
secretarial aid is furnished.	service for in- or out-patients)	

7. Anticipated Duration of Work:

One year (or more, depending upon results observed).

8. Facilities and Staff Available:

A laboratory equipped for neurovascular research, a physical therapy department and other hospital facilities are available for the investigation. Dr. Di Palma, Professor of Pharmacology, will offer suggestions and advice.

Participants: Dr. John Nodine, Assistant Professor of Medicine and Head of Section of Clinical Pharmacologic Research, has had long experience in neurovascular research in regard to the clinical use of drugs. Salem Lumish, M.D., Instructor in Medicine, has cooperated with Dr. Nodine in Pharmacologic Research in the Neurovascular Research Laboratory and Clinic.

10. Additional Information (Including relation of work to other projects and other sources of supply):

None.

/s/ Hobart A. Reimann, M.D.  
Director of Project

/s/ Edwin Robinson  
Comptroller

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#202

TOBACCO INDUSTRY RESEARCH COMMITTEE  
150 EAST FORTY SECOND STREET NEW YORK 17, N.Y.

Application For Research Grant

Date: April 4, 1958

*L. L. Rabb Acting Dir. of Project*

1. Name of Investigator: New position - Insect Pathologist (Tobacco).

2. Title: Insect Pathogens of Tobacco Insects.

3. Institution and Address: Entomology Department  
N. C. State College  
Raleigh, North Carolina

4. Project or Subject: Insecticides, which are toxic to humans and many animals, are used in huge quantities for the control of tobacco insects, principally hornworms and budworms. Residues of these insecticides have been found in tobacco smoke "consumed" by the American Public and have been considered among the possible causal factors of lung cancer.

Insect pathogens, most of which are harmless to man, often drastically reduce insect populations. Research has shown that some of these micro-organisms can be mass-produced and applied as sprays or dusts for effective control of certain pests. Rabb and his co-workers have demonstrated that hornworms can be successfully controlled with Bacillus thuringiensis, a pathogen harmless to man. Two commercial concerns are now in pilot-plant production of this organism, but additional research is necessary before a recommendation can be justified. Further progress is also dependent upon finding a pathogen or a combination of pathogens which will effectively control all of the major insect pests of tobacco. A search for such pathogens is the immediate objective of this project, whereas the long range objective is the elimination of insecticidal residues.

5. Detailed Plan of Procedure: Cultures of a number of insect pathogens are now available in other laboratories. Certain of these, selected on the basis of their performance against species closely related to tobacco pests, will be tested against tobacco insects.

Field collections of diseased insects, particularly budworms and hornworms, will be made and disease organisms isolated. These micro-organisms will be identified and cultured in sufficient quantities for laboratory screening against tobacco insects. Micro-organisms showing most promise will then be subjected to further study. Since considerable intraspecific variability in pathogenicity is known to occur, an attempt will be made to select for highest virulence in each pathogen cultured.

Attempts will then be made to develop techniques for mass-producing the most promising pathogens. Those which can be produced in sufficient quantities will be subjected to large-scale field tests, which will provide data for the ultimate evaluation of their potentialities as agents for use in applied control.

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6. Budget Plan:

	1st Year	2nd Year	3rd Year
Salaries	\$6,600	\$6,900	\$7,200
Expendable Supplies	1,000	500	500
Permanent Equipment	3,000	2,000	1,500
Overhead	---	---	---
Labor	600	2,000	2,200
Travel	600	400	400
Other	400	200	200
	\$12,200	\$12,000	\$12,000

7. Anticipated duration of Work: Three years.

8. Facilities and Staff Available: Office, laboratory, and insectary will be furnished by the Entomology Department of North Carolina State College. Plot land for field tests at several Tobacco Research Stations will be made available by the North Carolina Agriculture Experiment Station.

The project leader will have the close cooperation and counsel of the following entomologists now engaged in research on tobacco insects:

- R. L. Rabb, Entomology Department, North Carolina State College
- F. E. Guthrie, Entomology Department, North Carolina State College
- H. H. Neunzig, Entomology Department, North Carolina State College
- T. G. Bowery, Chemistry Department, North Carolina State College
- F. R. Lawson, U.S.D.A., Oxford Tobacco Research Station

9. Additional Requirements: None.

10. Additional Information (Including relation of work to other projects and sources of supply):

This project will be supported by concurrent work carried out under State Projects S-153, "Biology, Ecology, and Control of Insects Affecting Tobacco," and S-196, "Studies of the Biology and Ecology of the Corn Earworm and the Tobacco Budworm." The data obtained from these projects will greatly facilitate collection of diseased material, provision of insects for laboratory tests, and the execution of field tests.

The Laboratory of Insect Pathology, University of California, Berkeley, California, and a similar laboratory operated by the United States Department of Agriculture at Beltsville, Maryland, are actively engaged in developing microbial control of insect pests. Personnel of these laboratories have demonstrated a willingness to supply information and materials to other research workers. A short training period for the project leader at one of these laboratories might expedite the completion of the project.

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TOBACCO INDUSTRY RESEARCH COMMITTEE  
350 FIFTH AVENUE NEW YORK 1, N. Y.

Salaries  
Expendable Expenses  
Application For Research Grant  
Overhead  
Other

Date: October 20, 1954

37  
Smoking technique  
I'd think it would be  
difficult to control  
all important conditions  
see notes

1. Name of Investigator: Emanuel Revici, M.D.

For two years.

2. Title: Scientific Director Institute of Applied Biology has a physiological laboratory equipped for cancer research.

3. Institution & Address: Institute of Applied Biology, the part-time services of a 101 Lafayette Avenue, Brooklyn 17, New York. Laboratory facilities will be provided by our present staff.

4. Project or Subject: To determine whether tobacco smoke produces the nonspecific, abnormal metabolic pattern found by us in susceptible animals and humans, which may influence the evolution of pre-cancerous or non-invasive cancer cells or other abnormal tissues.

5. Detailed Plan of Procedure (Use reverse side if additional space is needed): The first phase of this study will be concerned with an attempt to determine whether laboratory animals exposed to tobacco smoke for long periods show the abnormal pattern described. Urine will be collected regularly from these animals before, after, and during exposure to tobacco smoke, and the pH and surface tension values determined. The second phase will try to determine whether the urine pattern of the animals of strains that have a high incidence of spontaneous cancer differ from those of low tumor strain animals, and whether evidence of an abnormal urine pattern is more easily brought about in susceptible animals when they are exposed to tobacco smoke. Finally, an attempt will be made to correlate urine changes and tumor development after exposure to tobacco smoke, in animals of a strain with high incidence of lung tumor.

In a later phase, we will carry out a more intensive study of the urine patterns in smokers and non-smokers in order to determine the existence of a relationship between this pattern and the incidence of cancer.

It should be noted that the two major procedures, pH and surface tension determinations are extremely simple. Simple colorimetric methods of determining pH can be used. Surface tension measurements are made with the Urotensiometer that we designed in connection with our cancer research program. With this simple device, accurate surface tension measurements can be performed in a few seconds. In addition, the method can be used in small laboratory animals, since only a few drops are required.

Signature: Emanuel Revici, M.D.  
Business Office of Institution

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The disturbed systemic metabolism that causes this abnormal urine pattern has been correlated to the presence of pathological foci. In studying the relationship between these metabolic patterns and disease, it has been found that such a local and general abnormal pattern can appear in the presence of an abnormal focus, and on the other hand, such a pattern induced experimentally can influence its evolution. Since these patterns can be produced through the pharmacological action of certain substances, they are able to significantly influence the evolution of existing abnormal conditions.

These patterns of metabolic imbalance have also been found in various abnormal conditions of experimental animals. We have studied the urine patterns in various strains of laboratory animals with and without tumors. A change to one of these abnormal patterns has been seen to occur while transplanted tumors were developing in the host. On the other hand, the tumor development was influenced in laboratory animals when one of these patterns was induced by the administration of various substances.

Normal individuals do not show these patterns. However, we have noted that the abnormal pattern described above has been encountered in some healthy individuals who are heavy smokers. Several substances present in cigarettes, experimentally bring about changes within abnormal tissues or systemically, corresponding to the pattern mentioned above. These include nicotine derivatives, glycerol, ethylene glycol and arsenic. These findings prompted us to propose a study to determine whether tobacco smoke can be shown to bring about nonspecific changes characterizing one of the patterns, and whether these changes influence the evolution of pathological conditions allegedly related to tobacco. The possibility of following these systemic changes through urinalysis, makes this study possible.

*what relation to cancer?*

*This I doubt*

If such a relationship is established, it will be of extreme importance. It will add to our knowledge of the mechanism of the pathogenic effects of smoking, and will help to identify one of the factors that may be involved in the pathogenesis of the clinically malignant neoplasms. It may permit the identification and ultimately, the elimination of the agent or agents in tobacco smoke that exert these nonspecific metabolic effects. On the other hand, it may explain why smoking affects only some smokers, and will permit the identification of these susceptible individuals by proper testing. If this is so, it may be possible to recognize them in time, through the existence of the analytical patterns, and to take special precautions to insure their safety, as is possible today in patients with Buerger's disease. It is quite possible that the information derived from the proposed study will also be found to have a bearing upon other conditions related to smoking.

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The disturbed systemic metabolism that causes this abnormal urine pattern has been correlated to the presence of pathological foci. In studying the relationship between these metabolic patterns and disease, it has been found that such a local and general abnormal pattern can appear in the presence of an abnormal focus, and on the other hand, such a pattern induced experimentally can influence its evolution. Since these patterns can be produced through the pharmacological action of certain substances, they are able to significantly influence the evolution of existing abnormal conditions.

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## 6. Budget Plan:

(for one year)

TOSACCO INDUSTRIES  
350 FIFTH AVENUE

Salaries	\$ 8,000
Expendable Supplies	1,500
Applied Permanent Equipment	1,000
Overhead	1,000
Other	500
Total	\$12,000

## 7. Anticipated Duration of Work: Annual Review, M.D.

One to two years.

## 8. Facilities and Staff Available:

The Institute of Applied Biology has a physiological laboratory equipped for cancer research.

## 9. Institution

Address:

Institution: In addition to the investigator, the part-time services of a tissue pathology technician, an animal care man, secretary, and clinical laboratory technician will be supplied by our present staff.

## 4. Project or Subject:

To determine whether tobacco smoke causes a metabolic pattern found in susceptible animals and humans.

## 9. Additional Requirements:

One full-time laboratory assistant.

## 10. Additional Information (Including relation of work to other projects and other sources of supply):

One of this study will be concerned with an attempt to determine whether laboratory animals exposed to tobacco smoke for long periods show the abnormal pattern described. Urine will be collected regularly from these animals before, after, and during exposure to tobacco smoke, and the pH and surface tension values determined. The second phase will try to determine whether the urine pattern of the animals of strains that have a high incidence of spontaneous cancer differs from those of low cancer strain animals, and whether evidence of an abnormal urine pattern is more readily brought about in susceptible animals when they are exposed to tobacco smoke. Finally, an attempt will be made to correlate urine pH and surface tension after exposure to tobacco smoke, in animals of a strain with high incidence of lung cancer.

In a later phase, we will carry out a more intensive study of the urine patterns in smokers and non-smokers in order to determine the existence of a relationship between urine pattern and the incidence of cancer.

It should be noted that the two major procedures, pH and surface tension determinations are extremely simple. Simple colorimetric methods of determining pH can be used. Surface tension measurements are made with the Protomembranes that are designed in connection with our cancer research program. With this simple device, accurate surface tension measurements can be performed in a few seconds. In addition, the device can be used in small laboratories.

Signature

Director of Project

/s/ Emanuel Revici, M.D.

/s/ Abraham Ravich, M.D.  
Business Officer of the Institution

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TOBACCO INDUSTRY RESEARCH COMMITTEE  
150 EAST FORTY SECOND STREET NEW YORK 17, N.Y.

#117 H2  
Activated 3/1/56  
Renewed 3/1/57

Application For Research Grant - Continuation for 1958-59  
and

Progress Report for 1957-58

Date: March 27, 1958

1. Name of Investigator: Dr. Pauline Heizer (Ph.D.)  
Name of grantee: Dr. Victor Richards (M.D.)
2. Title: Dr. Victor Richards, Professor of Surgery, Executive Head  
of Department of Surgery
3. Institution & Address: Stanford University School of Medicine  
Sacramento and Webster Sts., San Francisco 15, California
4. Project or Subject: A Comparative Study of the Effects of Whole and Fractionated  
Extracts of Cigarette Smoke and Those of Known Carcinogens on  
I. The Cytology and Nuclear DNA Content of Epidermis in Vari-  
ous Strains of Mice

5. Detailed Plan of Procedure (Use reverse side if additional space is needed):

Summary of Work Completed Thus Far

EXPERIMENTS (conducted to date)

Experiment I. (pilot experiment) Subdivisions I<sub>a</sub> & I<sub>b</sub>

Exp. I<sub>a</sub>. To test the effect of whole tobacco tar on the skins of mice. The animals used in this experiment consisted of a group of 24 C<sub>57</sub> Blacks (12 males and 12 females) obtained from the University of California (Berkeley) genetics laboratory. At the start of the experiment they were all approximately 2-1/2 months old.

Experimentals (Tar/ solvent A)

6 males and 6 females were painted every other day with one brushful (#5 brush) of whole tobacco tar in Solvent A. The whole dorsal region was covered. They were killed 21 days after the 1st application.

Controls (Toluene & 95% Alc)

6 males and 6 females were painted every other day with one brushful (#5) of 2 pts. Toluene to 3 pts. 95% alcohol. Killed at the same time as the experimentals.

The dorsal skin which had received the application in both experimentals and controls was tied to a piece of cardboard with a piece of nylon thread to prevent curling. Fixation in Lewdowsky's mixture and Bouin's.

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Tissues run up and sectioned, and DNA measurements are in progress.

Exp. I<sub>b</sub>. To test the effect of whole tobacco tar followed by Croton oil.  
Animals used - C<sub>57</sub> Blacks obtained from the same source as above.  
(12 males and 12 females)

Experimentals (Tar/Solvent A,  
followed by Croton Oil)

6 males and 6 females were painted every other day for 3 days with whole tar as above but after that with alternating applications of tar and 2.5% croton oil in acetone.

Killed 6 weeks after first painting

Skins treated as in Exp. I/a

Controls (Toluene 2 pts.  
to 95% Alcohol 3 pts.)

6 males and 6 females painted every other day with toluene and alcohol.

Killed at same time as exp.

Experiment II (pilot)

Here the Solvent A of the tar solution was evaporated with fans at room temperature and redissolved in pure acetone.

Exp. II<sub>a</sub>. To test the difference between acetone and toluene/alcohol as solvents for tobacco tar. Animals used - 6 males and 6 females of the same strain of mice as above (C<sub>57</sub>Bl)

Experimentals (Tar/Acetone)

3 males and 3 females painted as above with whole tar dissolved in acetone 1 pt. to 3 pts.

Killed 2 weeks after first treatment

Skin treated as in Experiment I

Controls (Acetone)

3 males and 3 females painted every other day with acetone only.

Killed at same time as experimentals

Exp. II<sub>b</sub>. To test the effect of tobacco tar dissolved in acetone followed by croton oil. Animals used - 6 males and 6 females of C<sub>57</sub> Blacks obtained from the same source as above.

Experimentals (Tar/Acetone  
followed by croton oil)

3 males and 3 females painted every other day as before with tar dissolved in acetone followed by alternate paintings of tar and croton oil

Killed 25 days after first treatment

Controls (Acetone only)

3 males and 3 females painted every other day with tar dissolved in acetone.

Killed at same time as experimentals

Experiment III. To test the effect of Solvent A alone.

Sent to Ecusta Paper Corp. for Solvent A.

4 females C<sub>57</sub> Blacks were used in this short experiment to test the difference between Solvent A, and acetone.

Experiment IV. Subdivisions IV<sub>1</sub>, IV<sub>2</sub>, IV<sub>3</sub>, IV<sub>4</sub>, IV<sub>5</sub>, IV<sub>6</sub>, IV<sub>7</sub>, IV<sub>8</sub>, IV<sub>9</sub>, IV<sub>10</sub>

(The arabic numerals indicate the number of days of tar applications were used.)

Object of this experiment: To test the effect of whole tar dissolved in Solvent A after one, two, three, four, five, six, seven, eight, nine and ten days of daily applications of tobacco tar in Solvent A.

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Applications were made of 2 drops from a 22-gauge needle on a tuberculin syringe to the shaved backs of mice. Animals used - C<sub>57</sub> Blacks (males only) 30 altogether - 30 in each group.

Each group consisted of 20 experimentals  
5 controls - Solvent A only  
5 controls - treatment

The painted region was stretched by placing on filter paper.  
The following fixatives were used

For DNA measurements  
and histologic studies

Lavdowsky's  
Baker's formal - calcium

For cytological studies

Chromosomes - A<sub>15</sub> & B<sub>16</sub>  
Mitochondria - Flemming's followed  
by post-chroming  
Golgi apparatus - Flemming's followed  
by osmication  
Elftman's silver method

Each piece of skin previous to dehydrating was rolled up into a coil and embedded thus in order to facilitate and make possible the study of the entire length of the skin piece on the same slide.

We are now in the process of preparing these last tissues for analysis cytologically, histologically and microspectrophotometrically.

On completion of this work we would like to apply for additional funds to complete experiments B and C of the initial application. Experiments B and C are designed to test the promoting action of whole cigarette smoke extract and its fractions.

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6. Budget Plan:

**Salaries:**  
**Dr. Pauline Heizer** \$ 7,000  
**1 Technician** 3,600  
**1 Animal caretaker** 3,000  
**1 Part-time secretary** 1,500  
**\$15,100**

**Salaries**  
**Expendable Supplies**  
**Permanent Equipment**  
**Overhead (5.5%)**  
**Other**

**\$15,100**  
**2,000**  
**1,000**  
**900**  
**\$19,000**

Total

**\$19,000 for 1958-59**

7. Anticipated Duration of Work: **1 more year**

8. Facilities and Staff Available: **Stanford Surgical Research Laboratory**

9. Additional Requirements: **None**

10. Additional Information (Including relation of work to other projects and other sources of supply):

Signature /s/ Victor Richards, M.D.  
 Director of Project

/s/ W. B. Steers  
 Business Officer of the Institution  
 Dean, Graduate Division

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TOBACCO INDUSTRY RESEARCH COMMITTEE  
350 FIFTH AVENUE  
NEW YORK 1, N.Y.

117

Application For Research Grant

Expendable Supplies  
Permanent Equipment  
Overhead  
Other Travel

Date: Nov. 21, 1955

1. Name of Investigator:

Victor Richards, M.D.  
Assistant to be used  
interchangeably with Farner

Total for 3 years

2. Title:

Professor of Surgery. Executive Head of Dept. of Surgery

3. Institution

Stanford University School of Medicine

4. Address:

Sacramento & Webster Sts., San Francisco 13, California

5. A copy of the Stanford Medical School

6. A copy of the new floor to be converted into an animal room

4. Project or Subject:

A comparative study of the effects of whole and fractionated  
extracts of cigarette smoke and those of known carcinogens on lung and

I. The cytology and nuclear DNA content of epidermis in various strains  
of mice.

and/or II. The cytology and nuclear DNA content of lung and epithelium of the  
bronchial tree of mice and hamsters.

5. Detailed Plan of Procedure (Use reverse side if additional space is needed):

I. The cytology and nuclear DNA content of epidermis in various strains of  
mice. (This portion of the program would require at least three years

10. Additional information relating to work to other sources of funds

8. EXPERIMENTS ON EPIDERMIS. This work will be related to a project.

EXPERIMENT A. To test for carcinogenicity of cigarette smoke extract and the fractions  
thereof as compared to the effects of known carcinogens. Effect of tobacco  
extracts and carcinogens on such tissues will be studied.

1. Materials. If experimental changes can be demonstrated in the lungs and bronchi.

a) Experimental animals - four different strains of inbred mice.

1. CAF<sub>1</sub> - the strain used by Wynder et al (Canc. Res. 13 (12) 855 (1953)). Since

b. The efforts of British investigators along the same lines have given  
abstain negative results to date (Brit. Emp. Canc. Camp. 1954) it is felt that  
both the work should be repeated with Wynder's strain using the extracts.  
disprovided by the T.I.R.C. on such tissue could be studied histologically

2. C<sub>3</sub>H (Strong) and microspectrum spectrometrically (DNA).

3. C<sub>57</sub> black (Little)

4. I

b) Substances to be tested.

1. Whole condensate from composite cigarette smoke, to be supplied by the T.I.R.C.

2. Five fractions of this extract, AB, EA, J, K, M, - separated according to the  
method of Kosak (Proc. Amer. Assoc. Canc. Res. 1 (2) 27 (1954)). If the T.I.R.C.  
would prefer to prepare these fractions then we could obtain them from this  
source.

3. Known carcinogens a. 20 methylcholanthrene

b. 3:4 benzpyrene

Signature of Victor Richards, M.D.

A. E. Brandin  
Business Officer of the Institution

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## 2. Method

- a) Treat skins of mice with increasing strengths of whole extract of smoke, fractions thereof and known carcinogens, 20 methylcholanthrene and 3:4 benzpyrene
- b) Controls would consist of  
mice painted with solvents alone  
untreated animals
- c) Since according to Smith et al. (Proc. Amer. Assoc. Canc. Res. 1, 45 (1954)) early skin changes such as loss of hair within 10 days and nucleolar enlargement within 4 days correlate well with the carcinogenicity of compounds applied to the skins of mice a comparative study would be carried out on the  
histology  
cytology  
nuclear DNA content (microspectrophotometrically measured)  
during these early stages.
- d) Similar studies would also be carried out at certain chosen later stages.
- e) All other organs in the body would be examined at autopsy for evidences of tumor formation.

EXPERIMENT B. To test for the initiating action of cigarette smoke extract and fractions thereof.

### 1. Materials.

- a) Experimental animals.  
The same four strains of mice as in experiment A.
- b) Substances to be tested.  
The same whole condensates from composite smoke of cigarettes and fractions thereof as in experiment A.

## 2. Method

- a) Make weekly applications of solutions of
  1. whole extract from cigarette smoke
  2. fractions of the above extract (especially fraction K, since positive results have been reported by Smith et al. (Proc. Amer. Assoc. Canc. Res. April 1954 )) - followed by a known promoter such as croton oil.

#### Groups of animals.

- a. In this group apply the whole extract in near maximal tolerated dose followed after an interval by repeated applications of croton oil.
  - b. Do the same with each fraction of the extract, using a separate group of animals for each.
  - c. Repeat a & b applying the test substances and croton oil alternately during part or all of the treatment period.
  - d. Treat this group with croton oil alone.
  - e. Controls treated with solvents alone.
  - f. Untreated controls.
- b) Compare results histologically, cytologically and microspectrophotometrically (DNA) in a, b, and c with those in d, e and f.

EXPERIMENT C. To test for promoting action of whole cigarette smoke extract and fractions thereof - CO-carcinogenesis.

### 1. Materials

- a) Experimental animals. The same four inbred strains of mice as in A and B.
- b) Substances to be tested. The same whole extract of cigarette smoke and fractions as in experiments A and B.

## 2. Method

- a) After an initial treatment with a known initiator such as 3:4 benzpyrene make weekly applications of
    1. whole extract
    2. fractions of the extract
- #### Groups of animals.
- a. In this group an initial treatment with 3:4 benzpyrene will be followed with weekly applications of whole smoke extract.

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Groups of animals (cont'd)

- b. The same will be done here with each fraction of the extract, using separate groups for each.
  - c. 3:4 benzpyrene alone
  - d. controls treated with solvents alone
  - e. untreated controls.
- b) Results in a, b, and c will be compared with those in d and e histologically, cytologically and microspectrophotometrically (DNA)

RESULTS IN EXPERIMENTS A, B, AND C WILL BE COMPARED.

The following experiments on lung and bronchial tree have been outlined as a possible alternative project in case the experiments on epidermis are too similar to projects being worked on by others. Or upon completion of project I this work could be continued for another three years.

II. The cytology and nuclear DNA content of lung and epithelium of the bronchial tree of mice and hamsters.

1. Materials.

a) Experimental animals.

- 1. Three strains of inbred mice with differing susceptibility to lung tumors.
  - a. Strain A (Strong) extremely susceptible
  - b. " C<sub>3</sub>H " intermediate susceptibility
  - c. " C<sub>57</sub> black (Little) low "
- 2. Hamsters

b) Substances to be used.

- 1. Whole extract of cigarette smoke (supplied by the T.I.R.C.)
- 2. Fractions thereof-separated according to the method of Kosak (preferably supplied by the T.I.R.C.)
- 3. Known carcinogens-20 methylcholanthrene and 3:4 benzpyrene

Experiments with mice. A comparative histological, cytological and microspectrophotometric (DNA) study will be made of the effects of cigarette smoke extract, its fractions and of known carcinogens after introduction of these substances into the respiratory tracts of the three strains of mice using the two following methods

1. Method of T.D. Day (Brit. Emp. Canc. Camp. 1954). This is a modification of the method of Magnus (J. Path. & Bact. 49, 21 (1939)).

Introduce the test substance (dissolved in a suitable solvent) on a blunt serum needle into the animal's mouth and then pass the tube into the esophagus and hold it there for a few seconds. This method was used by Day in preference to submitting the animals to aerosols of the tobacco tar as the latter method failed to result in effective concentrations in the lungs.

2. Method of Advervont. (U.S. Publ. Health Rep. 52, 1584 (1937)).

After anesthetizing the animal by the intraperitoneal injection of nembutal a fine thread saturated with the test substance is then drawn into the chest cavity by means of a fine needle passed between the ribs. The thread is then left within the lung. The mice are then sacrificed at chosen intervals and the lungs found to contain the threads (in some cases the lungs are missed) are studied as indicated above.

(Andervont was able to produce not only adenomas and adenocarcinomas with this method but also squamous cell carcinomas - the type associated with cigarette smoking in man)

3. Controls in both methods would consist of animals treated similarly but without either smoke extract, its fractions or the carcinogens.

Experiments with hamsters. A comparative histological, cytological and microspectrophotometric (DNA) study will be made of the effects of whole extract of smoke, its fractions and of known carcinogens by directly instilling these substances into the left bronchus of hamsters.

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permanent equipment  
 1 research microscope \$2,000  
 1 microspectrophotometer complete with Leitz Panphot micros.  
 U.V. light, Farrand photometer & monochromator \$6,900

1 full time technician - \$3,600  
 1 animal caretaker 3,000  
 1 full time Ph.D. (cytologist & cytochemist) 6,000  
 1 part time secretary 1,500  
 \$14,100

6. Budget Plan:

Applicable for Research Grant		1 Yr.	3 Yrs.
Expendable Supplies		\$14,100	\$42,300
Permanent Equipment		2,000	6,000
Overhead		1,000	3,000
Other		900	2,700
Travel			3,000
<b>Total for 3 yrs</b>			<b>\$65,900</b>

7. Anticipated Duration of Work: For project I - three years. If continued with project II - another 3 years.

8. Facilities and Staff Available:
 

- a. an entire laboratory to be used exclusively for this work in the dept. of surgery of the Stanford medical school
- b. a small room on the same floor to be converted into an animal room

9. Additional Requirements:
 

- a. a comparative study of the effects of whole and fractionated tobacco extract on the cytology and nuclear DNA content of epidermis in various strains of mice.
- b. The cytology and nuclear DNA content of lung and epithelium of the bronchial tree of mice and hamsters.

10. Additional Information (including relation of work to other projects and other sources of supply):
 

- a. Relation of work to other projects: The work could be related to a project to be launched in tissue culture in the dept. of surgery in which human lung tissue would be grown in hamster's cheek pouches. The direct effect of tobacco extracts and carcinogens on such transplants could be studied.
- b. Relation of work to other sources of supply: Fetal lung tissues could be obtained from this dept. (surgery) which could be cultivated in vitro (watch glass method) in the tissue culture lab on the same floor of the medical school. The direct effect of smoke extract on such tissue could be studied histologically cytologically and microspectrophotometrically (DNA).
- c. C57 black (Little)
- d. Substrates to be tested:
  - 1. Smoke condensate from composite cigarette to be supplied by the T.I.R.C.
  - 2. Five fractions of this extract, A1, A2, A3, A4, A5 - separated according to the method of Kosak (Proc. Amer. Assoc. Cancer Res. 1 (2) 27 (1954)). If the T.I.R.C. would prefer to prepare these fractions they could obtain them from this source.
  - 3. Known carcinogens
    - a. 20 methylcholanthrene
    - b. 3:4 benzopyrene

Signature: s/ Victor Richards, M.D.  
 Director of Project

s/ A. E. Brandin  
 Business Officer of the Institution  
 Business Manager

1003540853

STANFORD UNIVERSITY SCHOOL OF MEDICINE  
STANFORD UNIVERSITY HOSPITALS  
Clay and Webster Streets  
San Francisco 15, California

November 21, 1955

Tobacco Industry Research Committee  
350 Fifth Avenue  
New York 1, New York

Gentlemen:

I am anxious to send you additional information concerning our application for a research grant from the Tobacco Industry Research Committee. Our request is to study the effects of whole and fractionated extracts of cigarette smoke and those of known carcinogens on

- 1) The cytology and nuclear DNA content of epidermis in various strains of mice and
- 2) The cytology and nuclear DNA content of lung and epithelium of the bronchial tree of mice and hamsters.

This work would be conducted in the Surgical Laboratories of Stanford University School of Medicine, of which I am Professor of Surgery and Chairman of the Department. My main field of interest is thoracic surgery and previous research activities have been directed along these lines.

In order to enable us to approach in sound fashion a problem of this type, we have taken into the department as a research associate Dr. Pauline Heiser, who is a Ph.D. in Cytology and Cytochemistry. We have obtained from private donors approximately \$7,000 for research in the cancer field, which we are using to equip a laboratory and animal room for her. She would be the full time cytologist and cytochemist attached to this project and would be responsible with me for its execution. We would see to it that Dr. Heiser is not isolated completely in our laboratory, but would have ample time for conferences, lectures and attendances at other areas throughout the University so that she would stay skilled in her own field and gather additional strength and information for the completion of this work. If the research application is granted, this would be a long term project in our laboratory over the next three to five years.

There are two additional advantages which would accrue from this grant:  
1) The availability of Dr. Heiser in our laboratory will enable us to pursue more intently and profitably activities in the field of tissue transplantation and the study of embryonic tissues. 2) If functional changes can be demonstrated in the bronchial tree or lungs of animals receiving carcinogens, a correlation could be made between these functional changes of a morphological nature and the physiological changes that result. We have been interested in pulmonary function studies over the past few years and the work in the two fields could be correlated if morphological changes are produced in the lungs.

I hope that this additional information concerning our hopes and aspirations in the total departmental picture will be of help to you in considering our request for a research grant.

s/ Victor Richards, M.D.

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TOBACCO INDUSTRY RESEARCH COMMITTEE  
150 EAST FORTY SECOND STREET NEW YORK 17, N. Y.

Application For Research Grant

Date: October 9, 1958

1. Name of Investigator: J. Alfred Rider, M. D., PhD.
2. Title: Assistant Professor of Medicine; Assistant Chief, Gastrointestinal Clinic,  
Department of Medicine.
3. Institution & Address: University of California Medical Center  
San Francisco 22, California
4. Project or Subject: The Effects of Tobacco Smoke on Gastric Secretion

We propose to do a thorough study to determine whether or not the tobacco smoke from cigarettes increases gastric acidity, gastric pepsin, or gastric uropepsin. We would also like to clinically evaluate ulcer patients to determine whether their ulcers were aggravated by smoking.

Included in this study would be a comparison of the effects on gastric secretion of two major brands of filter and non-filter cigarettes.

5. Detailed Plan of Procedure (Use reverse side if additional space is needed):

A group of 10-20 patients without organic disease and a group of 10-20 patients with gastric or duodenal ulcer would be used for each brand of cigarettes with and without filters. After a basal gastric analysis, the patients will be given 4 cigarettes to smoke during the next hour, and during this time the gastric analysis will be continued. At the conclusion of this hour, the patients will not smoke any more cigarettes, but the gastric analysis will be continued for an additional hour to see if there are any delayed effects. It will, of course, be important to have control studies done to be sure that merely the presence of the tube itself is not a stimulant.

We would also compare the basal gastric analysis of approximately 100 smokers with that of 100 non-smokers.

In addition, 50 ulcer patients would be evaluated clinically to determine whether their symptoms were aggravated by smoking cigarettes. A comparison in these patients would be made between filter and non-filter cigarettes.

(cont'd)

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## 5. Detailed Plan of Procedure (cont'd)

### Procedures

#### 1. The exact procedure of the gastric analysis is as follows:

Patients are instructed to fast after 9 p.m. on the day preceding the test. On the morning of the test a Rehfuß tube is passed into the stomach. If there is any question as to the proper location of the tube, it is checked fluoroscopically. The residual gastric juice is aspirated and put into a specimen bottle marked "residuum." The patient is shown how to collect the gastric secretions and is instructed to expectorate his saliva so that it will not contaminate the gastric juice. He is then assisted in continually aspirating his stomach contents over the next hour. The basal-hour collection consists of four specimens taken at 15-minute intervals.

The gastric juice from each 15-minute period is measured for volume and titrated for free acid to the salmon pink end-point of Topfer's reagent, using 0.1 normal sodium hydroxide. The results are expressed in clinical units -- clinical units being the milliliters of 0.1 normal sodium hydroxide required to neutralize 100 milliliters of gastric juice. Milligrams of  $\text{HCl}$  are calculated as follows: clinical units  $\times$  volume in ml.  $\times$  0.365.  $\text{Mg. HCl/hr.} = \text{sum of mg. HCl of the four 15-minute specimens.}$

#### 2. The technique of gastric pepsin is as follows:

Determinations are performed on aliquots of 1-hour collections of gastric juice by the method of West, Ellis and Scott, with certain modifications. The exact procedure is as follows:

A 2.0 aliquot of diluted gastric juice (from 1:10 to 1:100 as necessary) is placed in a test tube with 0.05 ml. of 0.2% aqueous methyl orange and 0.1 ml. of 2N hydrochloric acid. Additional hydrochloric acid may be required to bring the solution to a pH of 3 or less. (If this is done, a correction is made in the subsequent calculation for the change in volume.) The acidified gastric juice is placed in a water bath at 37° C. before use. After one hour, .05 ml. of the gastric juice is transferred to a second tube, the volume is brought to 1.0 ml. with distilled water, and 1.0 ml. of acetate buffer is added. The contents of the tube are mixed thoroughly; 0.5 ml. of milk-buffer solution is added and quickly mixed; the tube is then returned to the water bath.

Timing: The test is timed from the moment the milk-buffer mixture is added. After one minute the tube is removed from the water bath, tilted and shaken slightly; the thin film remaining on the walls of the tube is then examined for casein particles. The tube is examined in this manner approximately every 10 seconds. The end point is reached when a fine precipitate begins to form, and the time is recorded. If the end point is reached in less than 80 seconds or more than 240 seconds, the test is repeated using smaller or larger amounts of gastric juice and corresponding amounts of water to bring the sample to 1.0 ml. Aliquots of 0.01 to 1.0 ml. of gastric juice may be used. In the case of 1.0 ml. samples, the test is timed for 10 minutes before a negative report is given.

(cont'd)

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## 5. Detailed Plan of Procedure (cont'd)

Calculation: Results are expressed in units of gastric pepsin secreted per hour, one unit being equivalent to 0.26 ug. of crystallized pepsin (Armour). Results are calculated as follows:

$$0.1 \times \frac{V}{v_{24}} \times \frac{(100)^{1.32}}{(t)} = \text{Units per hour}$$

where

- V = total volume in milliliters of 24-hour
- v = amount of gastric juice used in test in milliliters
- t = time in seconds to the end point

3. Uropepsin: With the exception of the dilution factor, the same technic is used for determinations of 4-hour and 24-hour uropepsin excretions.

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6. Budget Plan:

Salaries  
Expendable Supplies  
Permanent Equipment  
Overhead 15%  
Other Consulting Fee\*

\$	10,000./
	1,000./
	1,950./
	2,000./
Total	\$ 14,950./

\*for evaluation of results and preparation  
of manuscript.

7. Anticipated Duration of Work:

One year.

8. Facilities and Staff Available:

Facilities: Beckman pH Meter, Model G  
2 water baths  
refrigerator  
Gastric Secretary Laboratory, U.C. Medical Center  
Collection Room, Gastrointestinal Clinic  
Gastrointestinal Clinic Patients  
Office space, office furniture, calculator, typewriter,  
telephone and index file.

9. Additional Requirements:

Budget breakdown:	Salaries	
	Research Fellow (M.D.)	\$4,000.00
	Technician	4,000.00
	Half-time secretary	2,000.00

10. Additional Information (Including relation of work to other projects and other sources of supply):

None.

Signature /s./ J. Alfred Rider, M.D.  
Director of Project

J. Alfred Rider, M.D., Ph.D.

Stanley C. Bateman  
Business Officer of the Institution

1003540858

TOBACCO INDUSTRY RESEARCH COMMITTEE  
150 EAST FORTY SECOND STREET NEW YORK 17, N.Y.

#157R1  
(\$157 activated 7/1/57)

Application For Research Grant

cf #72R1  
Activated 7/1/55  
Renewed 7/1/56

Date: April 7, 1958

1. Name of Investigator: R. H. Rigdon, M. D.
2. Title: Professor of Pathology
3. Institution & Address: The University of Texas Medical Branch - Galveston, Texas
4. Project or Subject: EFFECT OF TOBACCO TAR AND OTHER CARCINOGENIC AGENTS ON THE RESPIRATORY TRACT OF THE DUCK

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5. Detailed Plan of Procedure (Use reverse side if additional space is needed):

We have developed the technique within the past year by which we can put large quantities of carcinogenic agents into the respiratory tract of the duck. We have put as much as 270 ml of saline into the larynx of our adult ducks within an interval of two hours. By using tween 80 we can put a suspension of carcinogenic agents into the lungs that we formerly could not.

We are completing a study in which we are to establish how particulate materials leave the respiratory tract. This is important since the duck does not have lymphoid nodules like mammals and phagocytosis does not occur in the duck, as we know it in mammals.

Sodium fluorescein has been found to pass immediately from the respiratory system into the blood stream. This fluorescent material can be demonstrated in the liver, gallbladder and lumen of the intestinal tract. No doubt there are other agents that will be rapidly eliminated from the lungs in a similar manner.

We would use the above technique and put tobacco tar down into the respiratory tract and observe its effect on the lung tissue and also see if it can be followed into the liver and bile by the use of the fluorescent techniques we have developed.

We now have a group of ducks given large amounts of methylcholanthrene in the above manner. These will be observed for another year. We would like to repeat these studies using other carcinogenic chemicals such as benzpyrene.

If we find that nicotine produces a major problem in our experimental study, we will consider using some of the techniques of removing it from tobacco tars.

Additional information may be learned from this study referable to the reaction of the respiratory tract. Although such experimental studies may not answer specific problems in man, I do think we need to compare the effects of agents on the skin, in the gastro-intestinal tract and in the respiratory tract of a specific host.

It is this particular problem we have been developing during the past several years. We would like to continue this phase of investigation. What occurs in the skin may not occur in the lungs of the same animal. Our results so far support this opinion.

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6. Budget Plan:

Salaries	\$3,090.00
Expendable Supplies	1,200.00
Permanent Equipment	688.50
Overhead	300.00
Other	\$5,278.50
*This includes \$67.50 O.A.S.I. and \$22.50 W.C.I.	

7. Anticipated Duration of Work: **One year**

8. Facilities and Staff Available: **Dr. Rigdon's Laboratory**  
**Present staff adequate to carry on this problem**

9. Additional Requirements: **None**

10. Additional Information (Including relation of work to other projects and other sources of supply):

**The U.S. Public Health Service is continuing its grant to enable me to study cancer in the duck produced by carcinogenic agents.**

**Publications supported by the Tobacco Industry Research Grant during the past year:**

**Spontaneous Regression of Tumors Produced by Methylcholanthrene in the Skin of Fowls - published Scientific Proceedings of the First Pan American Cancer Cytology Congress**

**The Respiratory System in the Normal White Pekin Duck - submitted to The American Journal of Anatomy**

**Papers referable to smoking either published or accepted for publication during past year:**

**A Consideration of Smoking and Cancer of the Lung with a Review of the Literature. Southern Medical Journal 50:524-532, 1957**

**Smoking and Disease. A Study Based upon 12,050 Individuals. Texas Reports on Biology and Medicine 16:116-132, 1958**

**Cancer of the Lung from 1900 to 1930. A Historical Review. Accepted Surgery, Gynecology & Obstetrics, December, 1957.**

Signature /s./R. H. Rigdon  
Director of Project

/s./E. D. Walker  
Business Officer of the Institution

1003540861

TIRC Grant #157

R. H. Rigdon, M.D.  
The University of Texas -  
Medical Branch

Progress Report No. 1  
(See #72, #72R1, Reports  
No. 1, 2, 3.)

February 21, 1958

Effect of Tobacco Tar on  
Respiratory Tract of the Duck

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We have been interested in the past year in developing the respiratory tract as a specific system to study experimental cancer. This work, of course, has been carried out in the white Pekin duck. I feel that we have accomplished a good deal during the past year in developing some technique for this study.

We now are able to put 50 to 100 cc. of solution into the respiratory tract of the duck without encountering any difficulty. This means that we can put a large amount of a carcinogenic agent down into the respiratory tract. It has required considerable time and effort to work out the normal respiratory tract in the duck and to find out what happens to liquids and particulate material when put into the respiratory tract. We have been able to demonstrate that sodium fluorescein will go immediately from the respiratory tract into the circulating blood; however, we cannot demonstrate methylcholanthrene in the liver and intestines forty-eight hours after we put approximately 250 mgs. of it into the respiratory tract although it is still present in the lungs and air sacs. This technique will enable us to compare the response of the respiratory tract to that of the skin. We do have ducks in which we put smaller amounts of methylcholanthrene into the respiratory tract. These have been under observation for almost a year and, as far as we know, none have developed tumors. Using this approach I think we can learn something of the elimination of chemicals from the respiratory tract. Such may throw some light on the problem of chemicals in the respiratory tract and their effects. This approach will enable us to use many other agents in large quantities in the respiratory tract, a technique which has not been used previously.

We have two manuscripts based upon the foregoing just about ready for publication.

As you may recall, we put tobacco tar into the respiratory tract of a small number of ducks for a period of about three months. Six or eight months later we sacrificed some of these birds but did not find anything of interest in the lungs other than some pigment in the lymphoid tissue. I have not observed any cellular changes or anything to suggest a neoplasm. We have three or four of this group of ducks still under observation. It is now about a year and a half since this tobacco tar experiment was started. I expect to sacrifice these birds and examine them within the next three or four months.

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I think it would be advisable to study the effects of chemicals on the respiratory tract of ducks and in this group to use some tobacco tar. The only problem that presents itself at this time is that the presence of nicotine in the tobacco tar may prove injurious to the ducks. I base this upon our other studies with tobacco tar. If there is any known way in which the tobacco tar can be treated to remove the nicotine, then the residue could be put into the respiratory tract in large amounts. I believe such a study would be advantageous in pursuit of this problem.

We will be able to complete some of the studies that we have been making over the past two years within the next six months as far as I see it now. I hope the Tobacco Industry Research Committee will be able to continue their assistance to me, enabling us to develop some of the basic problems in the field of carcinogenesis and lung lesions. I will be happy to receive an application form for a grant for the coming year.

You may be interested in knowing that the paper that Mrs. Kirchoff and myself worked on for a long time, correlating the smoking habits of different individuals with different disease processes, will be published in Texas Reports within the next month or six weeks. The review of cancer of the lung from 1900 to 1930 has been accepted by Surgery, Gynecology and Obstetrics for publication.

Mrs. Kirchoff is working on the problem of compiling all available information on cancer of the lung referable to frequency and the frequency in the male and female; the latter, as everyone knows, is in a state of confusion. We are hoping that we can at least pull all of this data together in order that it can be better evaluated. We are still working on our references on lung cancer. I have not approached the American Cancer Society yet for some aid on this study. The reason I haven't is that we are snowed under with our other studies at the moment.

Please excuse this long discussion, but I did want to let the Tobacco Industry Research Committee know what we are doing in order that they can evaluate our problem and see if they are in a position to continue assistance to us.

\* \* \*

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6. Budget Max

Application for Renewal of Research Grant

Application For Research Grant

Permanent Equipment

Overhead

Other

Date:

For April 2, 1956

1. Name of Investigator:

R. H. Higdon, M. D.

2. Title:

Immunology

Professor of Pathology. Director, Laboratory of Experimental Pathology

3. Institution

& Address:

Laboratory of Experimental Pathology  
University of Texas Medical Branch  
Galveston, Texas

4. Project or Subject:

- Study effect of methylcholanthrene on tissues of the duck.
- Compare reaction in chicken and turkey.
- Complete study of the effect of methylcholanthrene on trachea.
- Culture tumor in yolk sac of developing chick embryos and on chorio-allantoic membrane of chicks.

5. Detailed Plan of Procedure (Use reverse side if additional space is needed):

The procedure for these studies will be similar to that used during the past several years. We wish to emphasize the problem of methylcholanthrene in the trachea of the duck. It is necessary to observe these birds over a period of twelve to eighteen months.

In view of the spontaneous regression of these experimentally induced tumors in fowl, attempts will be made to culture these tumors in the yolk sac and on the chorio-allantoic membranes. This latter technique has been developed by Dr. Taylor in Austin, Texas. It will be duplicated by us. We should be cautious in concluding what might occur in the trachea as a result of a suspected carcinogen. As I saw it, it is necessary to study the effect of the specific carcinogen in the specific tissue under consideration. The transferring of information from one species to another is dangerous, in my opinion. The scientific demonstration of these results, I think would be important in any study of the etiology of cancer.

Signature

Robert H. Higdon

Business Office of the Institution

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6. Budget Plan:

Salaries	\$ 2,800.00
Expendable Supplies	1,000.00
Permanent Equipment	500.00
Overhead	600.00
Other	300.00
Total	5,200.00

1. Name of Investigator:

R. E. RIGDON, M. D.

7. Anticipated Duration of Work:

2. Title:

Two years  
Investigation of Methylcholanthrene

8. Facilities and Staff Available:

Director, Laboratory of Experimental Pathology

3. Institution:

4. Address:

Laboratory of Experimental Pathology  
Calvin Present staff adequate to carry on this problem  
Galveston, Texas

4. Project or Subject:

6. Study effect of methylcholanthrene on tissues of the duck.  
11. To determine relation in chicks and embryo.  
9. Additional Requirements:  
12. Study of the effect of methylcholanthrene on tumours.  
13. Subject tissue in which one of developing chick embryos and on  
characteristically occurrence of tumours.

5. Detailed Plan of Procedure (Use reverse side if additional space is needed):

10. Additional Information (Including relation of work to other projects and other sources of supply):

The procedure for these studies will be similar to that used during the past years. The United States Public Health Service is continuing their assistance in these studies. We have an additional request to the United States Public Health Service to enable us to make some changes in our laboratory so that tumors may be cultured on the chick embryo. Our observations on the different response of different tissues in the duck to methylcholanthrene are important in the etiology of tumors. Different tissues respond differently to the same carcinogen. In view of this fact, one should be cautious in concluding what might occur in one tissue or another by a suspected carcinogen. As I see it, it is necessary to prove the effect of the specific carcinogen in the specific tissue under consideration. The transferring of information from one specie to another is dangerous, in my opinion. The scientific demonstration of these points, I think would be important in any study of the etiology of tumors.

Signature

Director of Project RIGDON

Business Office: WALKER Institution

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#72  
COPY

THE UNIVERSITY OF TEXAS - MEDICAL BRANCH  
Galveston

May 18, 1956

Robert C. Hockett, Associate Scientific Director  
Tobacco Industry Research Committee  
150 East Forty Second Street  
New York 17, New York

Dear Dr. Hockett:

Thank you very much for your letter of May 9 informing me of the decision of the Committee to renew my appropriation for the coming year.

I am sorry about the paper, "Carcinogenesis in the White Pekin Duck." Medical News wanted a copy before it was presented at the M.D. Anderson Symposium; in our haste to get the paper off, we apparently crossed up on the addresses. I am glad that you finally got a copy.

In view of your questions about the overall problem of cancer of the lung, I will take this opportunity to state to you some of my opinions referable to this problem. In the first place I certainly agree with you that little can be learned concerning the etiology of lung cancer from experimental studies such as painting the skin of mice, etc. I think the important fact the experimental observations will show is that different tissues react differently to the same carcinogenic agent. Experimental studies with carcinogens, as far as I am concerned, may help to establish the basic mechanism of cancer and serve as a basis for observations on possible therapeutic agents - the latter will be merely a screening procedure.

I feel that experimental observations on metaplasia in the trachea of the duck may show us that metaplasia is not necessarily followed by neoplasia. Again, this principle may not hold true in man. However, from a review of the literature and from the material which I have studied, it seems to be definitely established that metaplasia may frequently occur in the respiratory tract of man without any evidence of neoplasia. I think it would be well worthwhile to establish, both in man and in experimental animals, the relationship of metaplasia to neoplasia. I feel that our studies on the trachea of the duck will be helpful along this line. I may say that at this time we have a good bit of information regarding these changes in the trachea. We have not observed anything that we would interpret as a malignant process.

With regard to a positive approach to this problem of lung cancer in man, I likewise find it difficult to devise any experiment that would throw light on the problem. Of course, I am of the firm belief that we have insufficient data to warrant the fact that cancer of the lung is actually increasing. I know that we have statistics that would suggest that this is occurring, but I find it very difficult to accept the cases on which the statistics are based. Personally,

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I feel that one of the major problems would be to establish whether or not cancer of the lung is actually increasing. If it is not increasing, we do not have a basic problem with regard to any etiologic agent, especially a product of tobacco. I have expressed this opinion previously and was interested in observing that some man from Baltimore published an article in the North Carolina State Medical Journal this month emphasizing this problem of the frequency of cancer of the lung. As you well know, there are many observations that question the actual increase of cancer of the lung. It is because of this belief that we have been so interested in compiling data referable to the frequency of cancer of the lung 25 to 50 years ago. At that early date there are many references to indicate that cancer of the lung was on the increase. The present attitude referable to the frequency of cancer of the lung, therefore, is now new. I personally feel that a review of previous work along this line would be profitable.

Certainly the clinical data on which the diagnosis of cancer of the lung was made 25 to 30 years ago must be looked upon today as inadequate. No doubt there were many more cases than were recorded. If this is true, the increase that is alleged to have occurred today is not a true increase. I know no way to establish this particular point.

I have been thinking of compiling the data on the frequency of tuberculosis and cancer of the lung in the same individual. This would emphasize that probably there were cases considered as tuberculosis that really could have been tuberculosis plus cancer of the lung. It seems to me that this back door approach to the problem might emphasize that probably the increase that we see today is not real.

Gilliam, at the National Institutes of Health, has made some statistical observations along this line. I believe he has stated that cancer of the lung increased from 1914 to 1930 but has not increased since then. This observation would support the idea that we are not seeing a real increase in frequency today. In this connection I was very much interested in a talk that Dr. Emma Moss, from Charity Hospital in New Orleans, recently made at the Texas Medical Association Convention. It is her opinion that cancer of the lung has not increased in New Orleans during the past seven years. This emphasizes the necessity of critically evaluating the bases for Dr. Ochsner's statements. It has been suggested that many of Dr. Ochsner's patients come from out of the city and probably some from out of the state. This naturally would cause him to see an increased number of cases. We have experienced the same situation here in Galveston. Our clinicians are impressed by the large number of cases of cancer of the lung they have been seeing. However, when we reviewed the county of origin of a group of these cases, we found that they were coming into this clinic from all over Texas. I certainly could not conclude that there was an actual increase in the number of cases of lung cancer merely because we are seeing more here in this institution. This situation is one that we were anxious to study during the coming year because we felt that it would help support an opinion as to whether cancer of the lung is actually increasing or whether it is only apparently on the increase.

X

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You can see from the above why I cannot get too enthused about any specific agent being responsible for cancer of the lung - I don't care whether it is smog or cigarette tars. A recent study that we made emphasized that cancer of the lung was on the increase in Texas where we do not have smog. I don't see how we can attribute the increase in California to the smog. We cannot agree with the opinion that cigarettes are causing this increase, based upon our recent study of the frequency of smoking and the observations now underway in which we are correlating different disease processes with the amount the patient stated that he smoked. As you may recall from our recent publication in the Journal of the National Cancer Institute, one pack of cigarettes a day is the average amount consumed by a smoker. Our observations so far with regard to disease processes show that many people that smoke have accidents, a high percentage have acute appendicitis, and we are very impressed by the correlation between smoking and benign tumors which occur in varying places of the human body. Personally, I don't think it sound to select two diseases, cancer of the lung and coronary occlusion, for a study referable to smoking. I think the data should include many other disease processes and this is what we are doing. I believe we will be able to publish this data in the near future although we do not have money to carry this on after July 1.

These approaches that I have mentioned are indirect; however, I do feel very strongly that they could contribute valuable information to the overall problem. If they are shown to be correct then much money and effort is being used to support opinions that are not now facts. This does not imply that I am not scientifically interested in the experimental approach to disease. I personally feel that the approach to cancer of the lung is a study of cancer: what is found out with regard to all cancer will help in this complicated problem.

Probably I should apologize for this long letter, but I interpreted your recent letter as a chance to get some of these thoughts out of my system. I hope that the opinions that I have expressed are not so far afield that it would be ill advised for me to discuss them when I go to Green Bay, Wisconsin the latter part of June to give a lecture.

Thanking you again for your interest and support in our work, I am,

Yours truly,

/s/ R. H. Rigdon, M.D.  
Professor of Pathology

RHR.k

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## TOBACCO INDUSTRY RESEARCH COMMITTEE

6. Budget Plan for one year

350 FIFTH AVENUE

NEW YORK 1, N. Y.

72

Salaries  
 Expenses  
Application For Research Grant

Overhead  
 Other

\$3,200.00  
 1,200.00  
 200.00  
 400.00  
 200.00

Date: Total  
 March 17, 1955

7. Name of Investigator: **R. H. Rigdon, M. D.**

2. Title: **Professor of Pathology and Director of the Laboratory of Experimental Pathology**

3. Institution & Address: **Medical Branch - University of Texas - Galveston, Texas**

4. Project or Subject:

Additional Information: **None**  
 Study the effect of methylcholanthrene on the tissues of the duck. To compare the effect of methylcholanthrene on different tissue with emphasis on the reaction in the trachea when compared with the skin of the body and the web of the foot.

5. Detailed Plan of Procedure (Use reverse side if additional space is needed): These studies have been supported for the past 3 years by the U.S. Public Health Service. The project for the coming year I have been studying this problem for 4 years now and have worked out the details for the study of the effect of methylcholanthrene on the skin of the duck. Several publications have been made on these results. We have a study about completed on the relation of trauma to the development of tumors in the skin following the local application of methylcholanthrene; trauma was produced by plucking the feathers. We have already obtained the tracheas from 50 normal ducks for controls in the study of the effect of methylcholanthrene on the trachea. Methylcholanthrene crystals have been put into the tracheas of 10 ducks, some of which have been observed now for 6 months and apparently no tumors are present. These ducks will be sacrificed later for histologic study. More methylcholanthrene can be put into the trachea in mineral oil and will cover a large area of the surface. Already I have been able to put 0.5 ml. of mineral oil down into the trachea without serious complications. One of a group of 10 ducks developed lipoid pneumonia. I believe this technique will prove satisfactory for the study of methylcholanthrene in the trachea. Other carcinogens may be studied in the duck for their effect on the trachea should this experiment prove satisfactory.

Signature: **R. H. Rigdon**  
 Director of Project

1003540869

6. Budget Plan: for one year

Salaries	\$3,200.00
Expendable Supplies	1,200.00
Permanent Equipment	250.00
Overhead	490.00
Other	250.00
Total	\$5,390.00

7. Anticipated Duration of Work:

R. H. Two years B.

8. Facilities and Staff Available:

Present Laboratory of Experimental Pathology Laboratory of  
Experiments Present staff adequate to carry on this problem.

9. Institution

& Address:

Medical Branch - University of Texas - Galveston, Texas

4. Project or Subject:

9. Additional Requirements:

None

Study the effect of methylcholanthrene on the tissues of the duck. To compare the effect of methylcholanthrene on different tissues with emphasis on the reaction in the trachea when compared with the skin of the body and the liver of the duck.

10. Additional Information (Including relation of work to other projects and other sources of supply):

These studies have been supported for the past 3 years by the U.S. Public Health Service. The project for the coming year (June 1, 1955) was approved but no funds are available. Because of this fact, other financial assistance is needed. Methylcholanthrene on the skin of the duck. Several A basic study of the reaction of duck tissue to carcinogens will be of value in any study of neoplasms. The following tumors have already been produced in the ducks: papillomas, hemangiomas, squamous cell carcinomas, lipomas and a variety of neurogenic tumors. In the chicken we have produced many squamous cell carcinomas and one hemangioma. The tissue reactions accompanying these tumors in the chicken are now under study. With a basic knowledge of the reaction of duck tissue to methylcholanthrene, we can then compare the reaction of the duck tissues to other carcinogens, such as those alleged to be in tobacco. This is especially true for referable to the respiratory tract. We may also see how far one is justified in predicting that since a carcinogen produced a cancer in one type of tissue it can be assumed that a similar lesion will occur in an unrelated type of tissue. The University supports our study with money for some personnel and a limited amount of supplies and maintenance. It is necessary to supplement these funds with outside grants in order to carry out the project indicated on the preceding pages. In the trachea. Other carcinogens may be studied in the duck for their effect on the trachea should this experiment prove satisfactory.

Signature /s/ R. H. Rigdon  
Director of Project

/s/ Earl Appleman  
Business Officer of the Institution

1003540870



TOBACCO INDUSTRY RESEARCH COMMITTEE

6. Budget Plan:

150 EAST FORTY SECOND STREET

NEW YORK 17, N.Y.

Salaries		3000.00
Expendable Supplies		\$1570.00
Application For Research Grant:	Social Security	37.00
	Workman's C.I.	(18.35) 17%
Overhead		300.00
Other		586.50
	Total	

Date: April 2, 1957

17. Name of Investigator: of R. H. Higon, M.D.

22. Title: Titles and Staff Available: Professor of Pathology - Director, Laboratory of Experimental Pathology

3. Institution & Address: University of Texas Medical Branch Galveston, Texas

4. Project or Subject:

9. Additional Requirements: None

Effect of Tobacco Tar on Respiratory Tract of the Duck.

5. Detailed Plan of Procedure (Use reverse side if additional space is needed): (or sources of supply):

The U.S. Public Health Service is continuing for two years their great Tobacco tar will be suspended in mineral oil and 0.5 cc. of this solution will be put through the external larynx of the duck daily for varying periods of time. At different times ducks so treated will be sacrificed and the trachea will be examined under ultra violet light for fluorescent substances and then sections will be taken for histologic study.

The above techniques have been used for a year - so we are familiar with the routine. Only of this type of experiment is one known. As will be seen to follow the problem of tumor formation following pulmonary.

One of the most important points I would like to follow would be to determine how long fluorescent material can be demonstrated in the trachea of the duck following discontinuation of tobacco tar. Methylcholanthrene disappears so rapidly from the trachea. I would like to see what happens to tobacco tar in the trachea of these birds. Apparently some chemical change occurs with methylcholanthrene.

The lungs from the ducks would be followed in view of the fact that preliminary observations have shown the presence of black, granular material that must be tobacco tar since we have observed it only in ducks receiving tobacco tar.

Director of Project

Our studies so far have shown that there is no problem in putting as much as 0.5 cc. of mineral oil daily into the trachea; however, the ducks will show evidence of nicotine poisoning if too much tobacco tar is given.

Business Officer of the Institution

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**TOBACCO INDUSTRY RESEARCH**  
**150 EAST FORTY SECOND STREET NEW YORK 17, N.Y.**

**6. Budget Plan:**

Salaries	3000.00
Expendable Supplies	1200.00
Applicable <del>Tobacco Industry</del> Social security	90.00
Overhead Workmen's C.I.	688.50) 17%
Other	300.00
Total	5278.50

Date: April 2, 1957

**7. Anticipated Duration of Work:** 2 years

**8. Facilities and Staff Available:** Laboratory of Experimental Pathology of Governmental  
 Present staff adequate to carry on this problem

**3. Institution:** Laboratory of Experimental Pathology of Governmental  
 Address: Laboratory of Experimental Pathology

**6. Principal or Subject:**

**9. Additional Requirements:** None

Effect of Tobacco Tar on Methylcholanthrene Study of the Duck.

**10. Additional Information (Including relation of work to other projects and other sources of supply):**

The U.S. Public Health Service is continuing for two years their grant for the study of the effect of methylcholanthrene on the tissues of the duck. We are beginning the study of the effect of methylcholanthrene on the gastrointestinal tract of the duck. It will be important to know whether the squamous epithelial cells in the esophagus will react to methylcholanthrene the same as will the cells in the skin of the body, web of the foot, and the trachea.

The epithelium in the trachea is columnar. The duck is nicely suited for the study of this type of epithelium in the trachea. We will be able to follow the problem of tumor formation following metaplasia.

One of the main problems which I would like to follow would be an experiment in the normal ducks and those given only mineral oil will serve as controls. In the experiments where methylcholanthrene is studied and also for the experiments where tobacco tar is put into the trachea. We plan to have in the 100 tracheas from normal ducks and 100 tracheas from the mineral oil study experiments.

The lungs from the ducks would be analyzed in view of the fact that preliminary observations have shown the formation of bronchogenic carcinoma that may be related to the tar which is put into the trachea.

It is hoped that we have shown that there is no problem in carrying the work on the effect of mineral oil daily into the duck trachea; however, the results will show whether or not the tar is related to the duck trachea.

/s/ R. H. Rigdon  
 Director of Project

/s/ E. D. Walker  
 Business Officer of the Institution

1003540872

6. Endorsements

**Abstract**

Date: April 2, 1956

1. Name of Investigator:  
2. Address: **Wm R. H. Rigdon, M.D.**
3. Title: **Professor of Pathology, Director, Laboratory of Experimental Pathology**
4. Institution & Address: **University of Texas Medical Branch  
Galveston, Texas**
5. Project or Subject: **Cancer of the Lung in all Hispanicized Studies at University, Galveston,  
in a one hospital, Galveston Southern Medical J., Vol 104, 1931. PP 77-81**
6. Detailed Plan of Procedure (Use reverse side if additional space is needed):
  - a. Compile all references pertaining to cancer of the lung.
  - b. Prepare paper on cancer of the lung between 1900 and 1930.
  - c. Prepare paper on cancer of the lung between 1930 and 1955.
  - d. Check cases of cancer of the lung so diagnosed in hospital with ultimate diagnosis as recorded on death certificates.
7. Detailed Plan of Procedure (Use reverse side if additional space is needed):

At this time we have approximately 4,000 references pertaining to cancer of the lung. We would like to complete this project which we feel is now 75 percent completed. At this time it is our plan to compile these references in a book form which can be published. In this project we would like to separate the papers pertaining to specific subjects, in order that the references would be of greater value to those interested in the field of cancer of the lung. In compiling these references we are obtaining all available publications throughout the world and checking the bibliographies. We are availing ourselves of all the standard works, such as the Surgeon Generals Index, Index Medicus, etc. We have been impressed so far by the important studies made by many investigators throughout the world on the subject of cancer of the lung.

We have compiled some of the data referable to cancer of the lung before 1900. This is published in an article entitled "Cancer of the Lung before 1900: A Historical Review" in Texas Reports on Biology and Medicine, 13: 933, 1955. We would like to compile the data from 1900 to 1930, and also from 1930 to 1955, in a similar manner. It is impressive to note ~~at~~ in the period from 1900 to 1930 the numerous studies referable to the increase of cancer of the lung and also the many reports on the etiology of cancer of the lung. In the period between 1930 and 1955 we are <sup>impressed by</sup> ~~impressed by~~ the tremendous number of publications and the continuation of the discussion as to whether cancer of

Business Officer of the Institution

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the lung is on the increase or not. Also, this is the period in which the emphasis has been placed on smoking as a cause of cancer of the lung. A knowledge of what has gone on before should be carefully evaluated.

It has been my opinion, and still is, that many cases of cancer of the lung have not been recognized clinically in the past. Furthermore, it is my opinion that the term "cancer" has been avoided by many physicians in filling out death certificates because of the stigma attached to anyone having cancer. These and associated factors have caused me, as a pathologist, to question the accuracy of our vital statistics referable to cancer of the lung. To study this problem, we would like to review the cases of cancer of the lung, so diagnosed in our institution, and, if death has occurred, to check the vital statistics to establish the cause of death as given on the death certificate. In compiling the cases of cancer of the lung from our institution over a period of ten years, we may be able to evaluate this problem. In my way of thinking, the rapid increase of cancer of the lung may be influenced by improved diagnoses and the fact that "cancer" as a cause of death has been used more frequently during the last several years on death certificates.

1003540874

6. Budget Plan:

Salaries	\$ 6,000.00
Expendable Supplies	1,200.00
Permanent Equipment	
Overhead	1,117.50
Other	250.00
Total	8,567.50

7. Anticipated Duration of Work: R. H. Rigdon, M.D.

2. One-half of this project can be completed within a year; however, the second half will require approximately two years for completion.

3. We have many records now in the department referable to these cases of cancer of the lung, namely of Texas Medical Branch.

Mrs. Helen Kirchoff who is familiar with the entire problem of lung cancer will carry on the large share of the particular problem. Previous publications from this laboratory will show what she has done along this line:

4. Frequency of Cancer of the Lung in all Malignancies Studied at Autopsy. Rigdon, R. H. and Kirchoff, Helen. Southern Medical J., 44: 506, 1951. RE OVER

9. Additional Requirements:

- Compile all references pertaining to cancer of the lung.
- Statistical study on cancer of the lung between 1900 and 1930.
- ~~Statistical study on cancer of the lung between 1930 and 1955.~~
- Check listed of cancer of the lung so diagnosed in hospital.

None to estimate diagnosis as recorded on death certificates.

5. Detailed Plan of Procedure (Use reverse side if additional space is needed).

10. Additional Information (Including relation of work to other projects and other sources of supply):

At this time we have approximately 4,000 references pertaining to cancer of the lung. Compiling the references on the subject of cancer of the lung may help to familiarize present day investigators with the tremendous amount of work that has been done in the past. This project we would like to separate into papers pertaining to statistical subjects. In order that the references would be of the statistical study will be of help in evaluating the problem of cancer of the lung. This is essential, in my opinion, when we are attempting to attribute the increase to various etiological factors. We are availing ourselves of all the standard works, such as the Cancer General Index, Index Medicus, etc. This study has been supported for the past three years by the American Tobacco Co. tigators throughout the world on the subject of cancer of the lung.

We have compiled some of the data referable to cancer of the lung before 1900. This is published in an article entitled "Cancer of the Lung before 1900: A Historical Review" in Texas Reports on Biology and Medicine, 13: 933, 1955. We would like to compile the data from 1900 to 1930, and also from 1930 to 1955, in a similar manner. It is impressive to note as in the period from 1900 to 1930 the numerous studies referable to the increase of cancer of the lung. We have the many reports on the etiology of cancer of the lung. In the period between 1910 and 1939 we are Signatures of R. H. Rigdon, member of the American Tobacco Co. and the continuation of the study to whether cancer of

Business Office of the Institution

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8. Facilities and Staff Available - continued

Accuracy of Death Certificates for Establishing and Frequency of Cancer as Shown by Autopsy. Rigdon, R. H. and Kirchoff, Helen. Texas Reports on Biology and Medicine, 9: 652, 1951.

A Consideration of Some of the Theories Relative to the Etiology and Incidence of Lung Cancer. Rigdon, R. H. and Kirchoff, Helen. Texas Reports on Biology and Medicine, 10: 76, 1952.

Frequency of Cancer in the White and Colored Races as Observed at Autopsy between 1920 and 1949 at the Medical Branch. Rigdon, R. H., Kirchoff, Helen, and Walker, Mary Lee. Texas Reports on Biology and Medicine, 10: 914, 1952.

Smoking and Cancer of the Lung - Let's Review the Facts. Rigdon, R. H. and Kirchoff, Helen. Texas Reports on Biology and Medicine, 11: 715, 1953.

Smoking Habits of College Students in Texas. Kirchoff, Helen and Rigdon, R. H. Texas Reports on Biology and Medicine, 12: 292, 1954.

Smoking Habits of 21,612 Texans. Kirchoff, Helen and Rigdon, R. H. To be published in J. National Cancer Institute, March, 1956.

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**CONFIDENTIAL**

TIRC Grant #235 (see Nos. 72, 157)  
R. H. Rigdon, M.D.  
The University of Texas - Medical Branch

Report No. 1  
(C.f. #72, 72R1, Reports  
No. 1, 2, 3. Also  
#157, Report No. 1)

July , 1959 to Jan. 1960

Study of the Effect of Tobacco Tar on the White Pekin Duck

During 1958 and 1959 we completed a group of experiments in which tobacco tar in mineral oil was put into the trachea of white Pekin ducks. This was accepted for publication by the Archives of Pathology in June, 1959; however, it has not as yet been published. The title of this paper is "The Effect of Tobacco Condensate on the Respiratory Tract of White Pekin Ducks." No tumors occurred in the respiratory tract following the intratracheal injection of tobacco tar.

Using the same technique as with the tobacco tar, we put methylcholanthrene in mineral oil into the respiratory tract of white Pekin ducks. Extensive metaplasia occurred in the trachea but no squamous cell carcinomas occurred. The same controls, using only mineral oil, and the trachea from normal ducks were used for both the methylcholanthrene and tobacco tar experiments. This latter experiment was published in the Archives of Pathology in November, 1959. The title was "Effects of Methylcholanthrene on the Respiratory Tract of White Pekin Ducks."

With the knowledge that methylcholanthrene could be suspended in Tween 80, we have carried out a series of experiments during the past year comparing the effect of different amounts of methylcholanthrene on the respiratory tract. This experiment is still in progress. However, a large number of neoplasms, both carcinomas and sarcomas, already have occurred in the respiratory tract of these ducks, Tween 80 given in equal amounts has not produced any tumors so far.

This phase of the study of the effect of methylcholanthrene on the respiratory tract, as far as I know now, will be completed by June, 1960. An exhibit, showing the experimental production of pulmonary neoplasms in the white Pekin duck, was shown at the meeting of the Southern Medical Association in Atlanta, Georgia, in November, 1959. During the presentation of this exhibit the question of cancer and tobacco frequently arose. Our experimental observations in the duck were discussed.

During our studies with methylcholanthrene, we have observed the occurrence of amyloidosis; also, three of the ducks previously treated with tobacco tar developed amyloidosis. In September, 1959, we began treating a group of ducks daily with tobacco tar suspended in Tween 80 (3 ml. tar and 27 ml. of a 1% solution of Tween 80). One milliliter is given intratracheally daily for five days each week. This experiment is now in progress and will be continued for about two more months. The ducks then will be observed for a year, at which time they will be killed and autopsied. This will give us an additional number of ducks given tobacco tar intratracheally and, too, it will give us an opportunity to determine whether amyloidosis has occurred in this group of birds given the tobacco tar.

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At the present time I am questioning the occurrence of amyloid as related to either methylcholanthrene or tobacco tar. This is due to the finding of amyloid in two ducks that did not receive either methylcholanthrene or tobacco tar. This phase of the problem of amyloidosis is being carefully studied at the present time.

We are now setting up an experiment in which the polycyclic hydrocarbons anthracene and phenanthrene will be put into the trachea of the duck. These birds will be observed for a year. The results of this experiment will be compared with methylcholanthrene and tobacco tar studies. I would like to continue studying the effect of other chemical carcinogens on the respiratory tract of the white Pekin duck during the next several years. With the technique we now have developed, I believe it will be profitable to investigate the effect of other agents on the respiratory tract. Our observations so far in the duck indicate that the relation of metaplasia to neoplasms is not a simple straightforward one. This approach may be productive for additional information on neoplasia.

In another study, supported by the National Cancer Institute, we are comparing the effects of methylcholanthrene on different tissues of the duck. Methylcholanthrene pellets have been put into the brain, muscle and abdominal cavity. We are now comparing the effects of methylcholanthrene given orally with those resulting from intratracheal injections. These studies will show whether or not a carcinogenic agent will always produce a neoplasm in all tissues of the same host.

This report will give you some indication of the progress of our experimental studies supported by funds from the Tobacco Industry Research Committee.

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TOBACCO INDUSTRY RESEARCH COMMITTEE  
150 East Forty-second Street  
New York 17, N. Y.

#235R1

Committee:

Dr. Kotin, Chm.  
Dr. Jacobson  
Dr. Reimann

Activated 7/1/59  
CF. #72  
Activated 7/1/55  
Renewed 7/1/56  
and #157  
Activated 7/1/57  
Renewed 7/1/58

Application for Research Grant

Application for Renewal of Research Grant

Date: January 19, 1960

1. Name of Investigator: R. H. Rigdon, M.D.
2. Title: Professor of Pathology
3. Institution & Address: The University of Texas Medical Branch  
Galveston, Texas
4. Project or Subject:

STUDY OF THE EFFECT OF TOBACCO TAR ON THE WHITE PEKIN DUCK

5. Detailed Plan of Procedure:

Tobacco tar is now being given intratracheally to 12 ducks. The tar is suspended in a 1% solution of Tween 80 (3 ml. of tobacco condensate and 27 ml. of Tween 80). One milliliter of this suspension is given daily five days each week. This experiment was started on September 9, 1959 and will continue until April, 1960. The ducks will be observed for one year thereafter and then killed. So far none of the birds have died.

Tween 80 has been given intratracheally to a group of ducks during the past year for a control. These ducks are still under observation. So far no tumors have developed with Tween 80.

Anthracene and phenanthrene in Tween 80 will be given intratracheally to ducks to compare their effect with that already obtained with methylcholanthrene and tobacco tar. These birds will be observed for one year before they are killed. All the ducks will be autopsied and their tissues studied histologically.

6. Budget Plan:

July 1, 1960-June 30, 1961	Salaries	\$3112.50*
	Expendable Supplies	1200.00
	Permanent Equipment	
	Overhead	461.00
	Other - Travel	300.00
	Total	\$5073.50 (1)

\* This includes \$90.00 O.A.S.I. and \$22.50 W.C.I.

(1) This is an estimate of the total budget needed for 1960-'61. It can be reduced by the amount of the uncommitted balance remaining from the present grant as of June 30, 1960.

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7. Anticipated Duration of Work:

One year

8. Facilities and Staff Available:

The same facilities we have had during the past several years

9. Additional Requirements:

None

10. Additional Information (Including relation of work to other projects and other sources of supply):

This experiment will enable us to evaluate the problem of amyloidosis in three ducks previously given tobacco tar and reported in a paper accepted for publication by the Archives of Pathology. Recent observations in the duck would indicate that amyloidosis may not be due to the effects of methylcholanthrene or tobacco tar, as previously suggested from our studies. It will be very important to establish scientifically the relation of tobacco tar to the occurrence of amyloid in our white Pekin ducks.

This experiment will give us additional observations on the effect of tobacco tar on the respiratory tract of the duck. In previous experiments we have found little, if any, effects produced by the tobacco tar in the respiratory tract of the duck.

The observations of the effect of anthracene and phenanthrene on the respiratory tract of white Pekin ducks will give us additional data referable to the effect of another carcinogenic agent on the respiratory tract.

The United States Public Health Service has already approved a grant for \$6,500.00 to enable us to continue our study of cancer in the white Pekin duck from June 1, 1960 to May 31, 1961. This study is related to the production of pulmonary tumors and the effect of methylcholanthrene on the other tissues of the duck, such as the brain, muscle, retroperitoneal and gastro-intestinal tract.

Publications supported by the grant from the Tobacco Industry Research Committee and published since July, 1959:

1. The Effect of Methylcholanthrene on the Respiratory Tract of the White Pekin duck. Arch. Path. 68: 578, Nov., 1959.
2. The Effect of Tobacco Condensate on the Respiratory Tract of White Pekin Ducks. Accepted by Arch. Path., June, 1959.

Exhibit: Pulmonary Neoplasms. An Experimental Study. Shown at the meeting of the Southern Medical Association, Atlanta, Ga., Nov., 1959.

/s./ R. H. Rigdon  
Director of Project

/s./ E. D. Walker  
Business Officer of the Institution

1003540880

**CONFIDENTIAL**

TIRC Grant #72

Progress Report #2

Dr. R. H. Rigdon  
University of Texas,  
Medical Branch

April 1, 1956

Study the effect of methylcholanthrene on the tissues of the duck. To compare the effect of methylcholanthrene on different tissue with emphasis on reaction in the trachea when compared with the skin of the body and the web of the foot.

---

The money in this grant has been utilized in our experimental studies along with a grant from the United States Public Health Service.

The primary investigation has centered around a study of methylcholanthrene in the white Pekin duck. Emphasis has been on the type of tumor produced in the white Pekin duck in different anatomical sites. We have been interested in comparing the type of response in one portion of the body with that in another. A summary of these observations may be found in my most recent manuscript "Carcinogenesis in the Duck," of which you have a copy.

A new approach that we are now developing is to study the response in the trachea of the duck to methylcholanthrene. Our problem here has been to introduce methylcholanthrene into the trachea. Approximately 18 months ago we blew some crystals of methylcholanthrene into the trachea. These localized only in the proximal 3 or 4 cms. Our second attempt was to put 15 mgs. of methylcholanthrene per 1 cc. of mineral oil into the trachea. One-half cc. of this can be placed into the trachea daily for 15 to 20 days. Some of the ducks showed obstruction to the trachea and died. So far the percentage of deaths has been small. We have 25 of these birds under observation and we would like to continue observing them for a period of one year. I plan to increase this number to 50. Gross and histologic examinations are being followed on these tracheas.

During the past year we have been determining the presence or absence of methylcholanthrene in the skin and in the viscera following varying intervals of time after the carcinogen is last applied. Our results, like those of others, would show that methylcholanthrene disappears very rapidly after coming in contact with duck tissues. This study on the trachea is being followed in a similar manner. We are also using an ultra-violet light to demonstrate the presence or absence of methylcholanthrene in the tissues. These studies appear significant in helping us to evaluate the problem of carcinogenesis. In this connection it might be said that we have not observed any tumors in the liver; however, extensive degenerative changes have occurred. This would suggest that probably a break-down product of methylcholanthrene injures the liver and this break-down product is non-carcinogenic for the liver. Since the type of degeneration in the liver is similar to that produced by certain paraffin oils we now have some birds injected with paraffin oil to observe the hepatic lesions.

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One of the most interesting features of our entire study has been the spontaneous regression of the hemangiomas and the squamous cell carcinomas. Other investigators have produced metastasizing squamous cell tumors with methylcholanthrene in fowl. This apparently resulted from a much longer application of methylcholanthrene than we have used in our experiments. We now have a group of birds under observation in which we are continuing to apply methylcholanthrene in an attempt to obtain metastasizing squamous cell tumors. If this can be done, then we will have the problem of determining the mechanism by which these earlier lesions regress. There seems to be considerable clinical interest in the spontaneous regression of neoplasms in man. I feel that our studies may contribute to this overall problem. I am hoping that funds may be obtained which will enable me to grow these tumors on the chorio-allantoic membrane of the chick embryo and in the yolk sac. If we are able in this attempt, it will open up an exciting field with regard to these experimental lesions we are producing.

The following list of publications will indicate what we have been doing during the past year. Paper No. 5 should be out in the March number of Cancer Research. Paper No. 2 has been accepted by the Archives of Pathology. Paper No. 3 will be published as a part of the M. D. Anderson Symposium in Texas Reports on Biology and Medicine. Paper No. 1 has been submitted to Cancer. Paper No. 4 will be submitted to Cancer Research within the next few days.

1. Rigdon, R.H., Jack Walker, and A.H. Teddlie: Hemangiomas: An Experimental Study in the Duck. Submitted to Cancer, March 13, 1956.
2. Rigdon, R.H.: Trauma and Cancer. An Experimental Study in the Duck. Accepted by the Archives of Pathology.
3. Rigdon, R.H.: Carcinogenesis in the White Pekin Duck. Given at symposium at M. D. Anderson Hospital, Houston, March 28-31, 1956. To be published in Texas Reports on Biology and Medicine.
4. Rigdon, R.H.: Tumors Induced in Skin without Follicles. An Experimental Study in the Duck. Ready to be submitted.
5. Rigdon, R.H. and Murray D. Hooks: A Consideration of the Mechanism by which Squamous Cell Carcinomatoid Tumors in the Chicken Spontaneously Regress. Cancer Research, to be published March, 1956.

We appreciate your financial assistance to us in our experimental work. We hope that the work we have already done will justify you in continuing this aid. If there is any additional information, we will be happy to supply you with it.

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TOBACCO INDUSTRY RESEARCH COMMITTEE

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150 E. 42nd Street New York 17, N.Y.

Application for Research Grant

#235

May 8, 1959

(Cf. #72  
Activated 7/1/55  
Renewed 7/1/56  
#157  
Activated 7/1/57  
Renewed 7/1/58)

1. Name of Investigator: R. H. Rigdon, M.D.
2. Title: Professor of Pathology
3. Institution & Address: The University of Texas Medical Branch  
Galveston, Texas
4. Project or Subject: Study of the Effect of Tobacco Tar on the White Pekin Duck.
5. Detailed Plan of Procedure:

In the study of the effect of tobacco tar on the respiratory tract of white Pekin ducks we gave nine ducks tobacco tar intratracheally in mineral oil for 130 times and observed amyloid in the liver of three. One of these birds died on the 242nd day after the first intratracheal injection and two were sacrificed on the 756th day.

I think this experiment should be repeated. We used 1 milliliter of cigarette tar and 19 milliliters of liquid petrolatum. When larger amounts of tar are given, the ducks show clinical evidence of nicotine poisoning. It would be wise to give this tobacco tar orally to some ducks to see if amyloidosis results. I say this because we have produced amyloidosis in ducks given methylcholanthrene orally.

Ducks given the tobacco tar will be sacrificed at varying intervals and histologic studies of the viscera will be made.

- |                 |                     |            |
|-----------------|---------------------|------------|
| 6. Budget Plan: | Salaries            | \$3105.00* |
|                 | Expendable Supplies | 1200.00    |
|                 | Permanent Equipment |            |
|                 | Overhead (10%)      | 460.00     |
|                 | Other               | 300.00     |
|                 | Total               | \$5065.00  |

\* This includes \$82.50 O/A/S/I. and \$22.50 W.C.I.

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7. Anticipated Duration of Work:

Two years

8. Facilities and Staff Available:

The same facilities we have had during the past several years.

9. Additional Requirements:

None

10. Additional Information (Including relation of work to other projects and other sources of supply):

Amyloid was first observed in 1955 in ducks treated with methylcholanthrene (R.H. Rigdon, Atypical cirrhosis in the duck produced by methylcholanthrene. Am. J. Path. 31:451-473, 1955). We recently have observed amyloid in the liver, spleen, adrenals, kidneys and thyroid of ducks given one large intratracheal injection of methylcholanthrene. This study was reported at Duke University in March of 1959. The manuscript is now ready to submit for publication.

Additional information on amyloidosis referable to pathogenesis would be valuable. Since it can be produced in ducks with methylcholanthrene and there is evidence that it will follow intratracheal injections of tobacco tar, we should establish the latter as a scientific fact. At the present time we know that large amounts of methylcholanthrene, when put into the respiratory tract, will produce neoplasms. Tobacco tar has not produced any tumors, but amyloidosis has occurred. Additional studies on tobacco tar and the production of amyloidosis may contribute to the basic knowledge of the agents that are carcinogenic.

Articles either published or recently submitted and aided by a grant from the Tobacco Industry Research Committee:

1. Keratoacanthoma. Experimentally induced with methylcholanthrene in the chicken. Arch. Dermat. 79: 139-147, 1959.
2. Mechanism of removal of fluid and particulate material from the respiratory tract of the duck. Arch. Path. 67:215-227, 1959.
3. Cancer of the lung - the sex ratio. A review of the problem. Texas Reports Biol. and Med. 17:29-48, 1958.
4. The respiratory system in the normal white Pekin duck. Poultry Sci. 38: 196-210, 1959.
5. The effect of tobacco tar on the respiratory tract of white Pekin ducks. Arch. Path. Submitted for consideration March 3, 1959.
6. Effects of methylcholanthrene on the respiratory tract of the white Pekin duck. Arch. Path. Submitted for consideration March 3, 1959.

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## Mechanism of Removal of Fluid and Particulate Material from the Respiratory Tract of the Duck

R. H. RIGDON, M.D., Galveston, Texas

The mechanism of absorption of fluids, foreign proteins, and particulate matter from the respiratory tract of mammals has been studied. Robertson,<sup>1</sup> in 1941, after reviewing the literature, pointed out that the principle means employed by the body for the immobilization and removal of particulate matter was phagocytosis by large amoeboid cells referred to as alveolar phagocytes or "dust cells of von Innes." These cells enter the lymphatics and pass to the lymph nodes. The rate at which carbon-laden macrophages migrate into the lymph channels is slow. Drinker and Field<sup>2</sup> thought that certain kinds of minute particles passed directly into the lymphatics without the intermediate step of phagocytosis.

In 1903, MacCallum<sup>3</sup> was very much interested in the mechanism of absorption of granular materials from the peritoneum. He commented:

... there has been for a long time a desultory discussion as to the manner in which various materials are absorbed from the peritoneum and even in the case of solutions it does not yet seem quite clear whether they are absorbed exclusively by the lymphatics or very largely by the veins. ... To explain the passage of these granules through the endothelial wall into the lumen of the lacuna, it seems necessary to suppose that the connections of the endothelial cells are so lax that the violent pumping action of the respiratory movement is enough to force material between them when they come to form the only obstruction to its entrance.

It would seem to be the consensus that direct absorption through the mucosa of the

larynx, trachea, and bronchi is slight. Little absorption occurs from the pulmonary alveoli, with the exception of water and simple crystalloids.<sup>4</sup> Colin,<sup>5</sup> in 1873, gave 25 liters of water intratracheally to a horse within a period of three hours without serious consequence. Winternitz<sup>6</sup> and Smith observed that isotonic saline in dogs was absorbed very rapidly from the alveoli. Absorption of foreign serum from the respiratory tract does occur in guinea pigs and dogs; however, the degree of absorption is minimal.<sup>7,8</sup> Drinker et al.<sup>8</sup> showed that horse serum was transferred in the dog directly from the alveoli to the blood.

The anatomic structure of the respiratory system in the duck is similar in many ways to that of mammals. However, there are certain fundamental differences that would suggest a possible variation in function. There are no lymph nodes in the duck. The deep lymphatics of each lung are drained by vessels which emerge with the pulmonary vein. The superficial lymphatics of the lung form a wide network on its lower surface. Vessels emerge from this latter network at its outer edge and join the lymphatics following the internal thoracic artery. These lymphatic channels commence as branches draining the abdominal muscles and the diaphragm. They receive the pulmonary lymphatics and branches from the ribs and intercostal muscles and then communicate with the anterior vena cava.<sup>9</sup>

Air sacs, of course, are characteristic anatomic structures in birds.<sup>10</sup> There are nine air sacs in the chicken and duck and seven in the turkey.<sup>10-12</sup> The total volume of these air sacs in the duck is probably four to five times greater than that of both lungs combined. All of the air sacs in the

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Department of Pathology, University of Texas Medical Branch.

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duck communicate directly with the larger bronchi, and some are continuous with the sternum, humerus, and vertebrae.

Little attention has been given to the mechanism of the removal of fluid and particulate matter from the respiratory tract of birds. In the present study isotonic saline, fluorescein sodium, saccharated iron oxide, India ink, liquid petrolatum, and Lipodium spores were put into the trachea of white Pekin ducks. Observations were made to determine how these substances were removed from the respiratory tract.

### Methods and Materials

White Pekin ducks varying in age from 1 month to 2 years were used. They were kept in small batteries in the laboratory during the time of the acute experiments and in an outside pen for the longer experiments. Food and water were available to them at all times. The intratracheal injections were made with a syringe and an attached small plastic catheter 5 cm. in length. The mouth was opened manually. When the external larynx was opened for inhalation, the catheter was inserted into the trachea for a distance of 2 to 3 cm. The catheter was quickly withdrawn when the injection was completed. Because the birds were held in an upright position with neck elevated for a brief

interval after completing the intratracheal injection, regurgitation was reduced to a minimum.

Isotonic saline was given intratracheally to two ducks. The quantity and the time when given are shown in Figure 1. A total of 320 ml. was given to one duck within an interval of two hours. Two hundred milliliters of this fluid was given within a period of 45 minutes. Six ducks were given a single intratracheal injection of 50 ml. of a 0.5% solution of fluorescein sodium in distilled water. Several samples of blood were removed from the leg veins during the first 15 minutes. Other specimens of blood were removed after 4 to 5 hours and after 18 to 24 hours. The blood was citrated and observed for fluorescence with a high-intensity long-wave ultraviolet light.\* Usually 4 ml. of blood was put into a test tube with 4 ml. of a 1.0% solution of sodium citrate. Fluorescence was observed after 12 to 24 hours. Control samples of blood were removed before the sodium fluorescence was given.

Twenty-five milliliters of a saccharated iron oxide solution,† containing the equivalent of 100 mg. of elemental iron, or 4 mg. per milliliter, was given intratracheally to one duck. This bird was killed 60 minutes later. A 12.0% concentration of India ink, 0.5 ml., suspended in liquid petrolatum was given intratracheally to 20 ducks. Five of

\* A. S. Aloe No. 52140 ultraviolet mineralight, high-intensity long-wave, 3,660 Å.

† Feojectin, Smith, Kline & French Laboratories, Philadelphia.

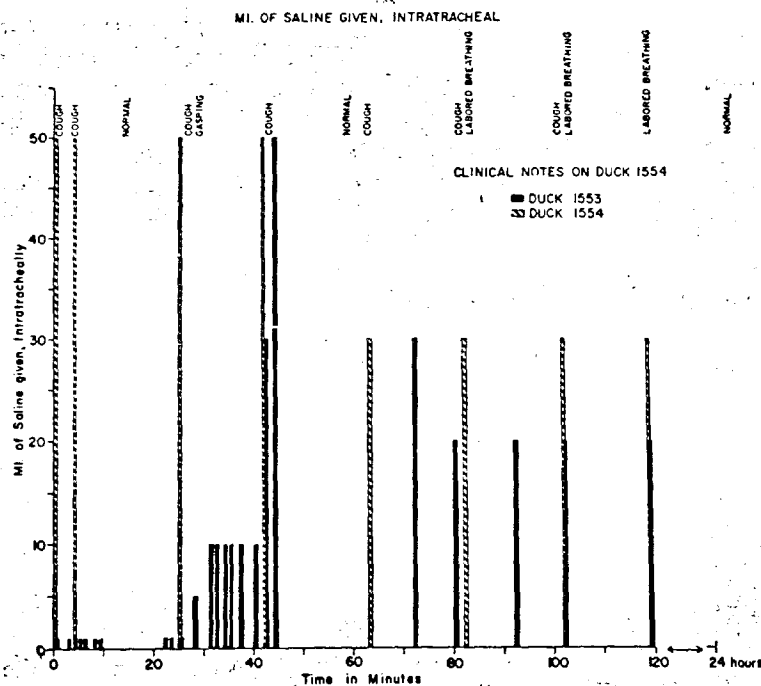


Fig. 1. — Intratracheal injection of saline in the duck. Notice the large amounts given within short intervals of time.



## RESPIRATORY TRACT OF DUCK

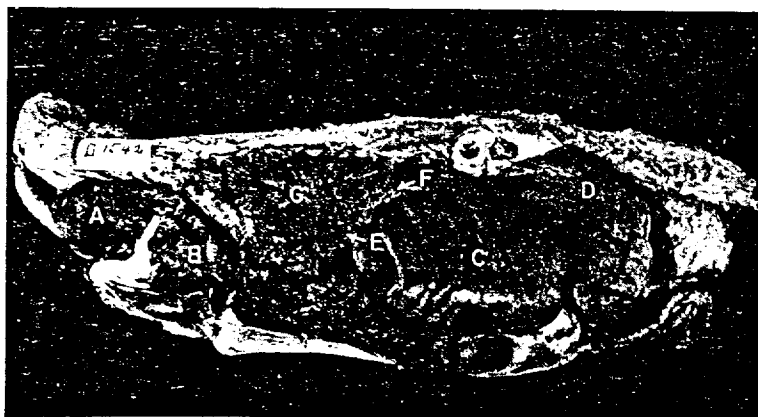
these were killed 5 to 15 minutes later; five, after 48 hours, and ten, after 65 hours. Ten additional ducks were given 0.5 ml. of the same suspension of India ink in liquid petrolatum daily except for Saturdays and Sundays for 10 days. These birds were killed 24 hours after the last intratracheal injection. Two ducks were given intratracheally 25.0 ml. of a 12.0% aqueous suspension of India ink and were killed five days later.

Liquid petrolatum was given intratracheally to 30 ducks. At each injection 0.5 ml. was given. Ten birds were given 10 daily injections except for Saturdays and Sundays and killed 24 hours after the last. Twenty birds were given 7 to 134 intratracheal injections and killed immediately or up to 18 days later.

All ducks in this experiment were killed by severing of the spinal cord. The ribs were cut on each side. The sternum was reflected onto the neck, permitting good exposure of the thoracic and abdominal viscera and the air sacs. This area then was observed under ultraviolet light for fluorescence in the lungs, air sacs, liver, gallbladder, and intestines. The trachea and lungs subsequently were removed intact. After the trachea was opened and the lungs were sectioned several times, all tissues were carefully examined for fluorescence. When the gallbladder was found to fluoresce, the bile was removed and put into the test tubes for further observations.

Sections were removed routinely from the proximal, middle, and lower third of the trachea and from both lungs. Other sections of tissue were removed for histologic study when gross changes were present. All sections were fixed immediately in a 4.0% solution of formaldehyde. Paraffin sections were prepared and stained routinely with hematoxylin and eosin. Select sections were stained with the periodic acid-Schiff reagent and with the Perl stain for hemosiderin. Osmic acid was used for the demonstration of lipids.

Fig. 2.—Air sacs in the duck after the intratracheal injection of latex. Air sacs: (A), cervical; (B), clavicular; (C), lesser abdominal; (D), greater abdominal. (E) shows the point of communication between the lung and the lesser abdominal air sac; (F) is the area of communication between the lung and the greater abdominal air sac; (G) is the lung.



Rigdon

## Experimental Data

### *Sodium Chloride in Trachea of Ducks.*—

These ducks were markedly dyspneic after receiving 100 to 150 ml. of saline (Fig. 1). Sometimes a small amount of fluid was sprayed from the external trachea. The birds soon returned to normal after the intratracheal injections of saline were discontinued. Forty-eight hours later both lungs were moderately hemorrhagic and slightly edematous. No fluid was present at this time in the air sacs (Fig. 2).

### *Fluorescein Sodium in Trachea of Ducks.*—

One adult duck was given intratracheally 50 ml. of fluorescein sodium within a period of two to three minutes. There was a minimum of respiratory difficulty immediately, but 10 minutes later the bird appeared normal. Five additional ducks were given intratracheally 50 ml. of fluorescein sodium. The degree of fluorescence in the blood progressively increased after the fluorescein sodium was given; the maximum intensity was reached 8 to 10 minutes after the injection. The blood removed four to five hours after the intratracheal injection of fluorescein sodium showed less fluorescence than the specimens removed after 10 to 15 minutes. There was no fluorescence in the blood that was removed 18 to 24 hours later. There was no fluid present in the air sacs of the ducks that were killed 18 to 24 hours after fluorescein sodium was injected intratracheally. A small amount of fluorescence, however, was

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present along the wall of the air sacs in three of the four ducks killed 18 to 24 hours after the intratracheal injection of the fluorescein sodium.

The gallbladder and portions of the small intestines fluoresced in the four ducks that were killed 18 to 24 hours after the intratracheal injection of the fluorescein sodium. When the bile was removed and put into a test tube there was considerable fluorescence. There was no fluorescence either of the bile or of the contents of the loop of the intestines in four normal ducks.

*Saccharated Iron Oxide in Trachea of Ducks.*—The respiratory tract and the lungs were markedly congested one hour after the intratracheal injection of this preparation of iron. A small amount of brownish-colored fluid was present in the air sacs. Histologic sections stained with hematoxylin and eosin showed a large amount of brown granular material within the lumen of the parabronchi, the air capillaries, and the air sacs.

A small amount of this brown-staining material was present in focal areas on the surface of the bronchial epithelium. The stroma beneath the epithelial cells lining the air sacs and the parabronchi was brownish-yellow as a result of the presence of this preparation of iron.

The brown granular material present in the hematoxylin-and-eosin-stained sections stained blue with the Perl reaction for hemosiderin (Fig. 3). A few small blue-staining areas were present within the layers of epithelial cells that line the trachea. The number of such foci was insignificant. Small clumps of blue-staining material were present within the lumen of some of the small lymphatic-like channels in the stroma between the lobules of lung tissue. Sometimes small collections of blue-staining material were present between the endothelial cells lining the lymphatic and vascular channels (Fig. 4).

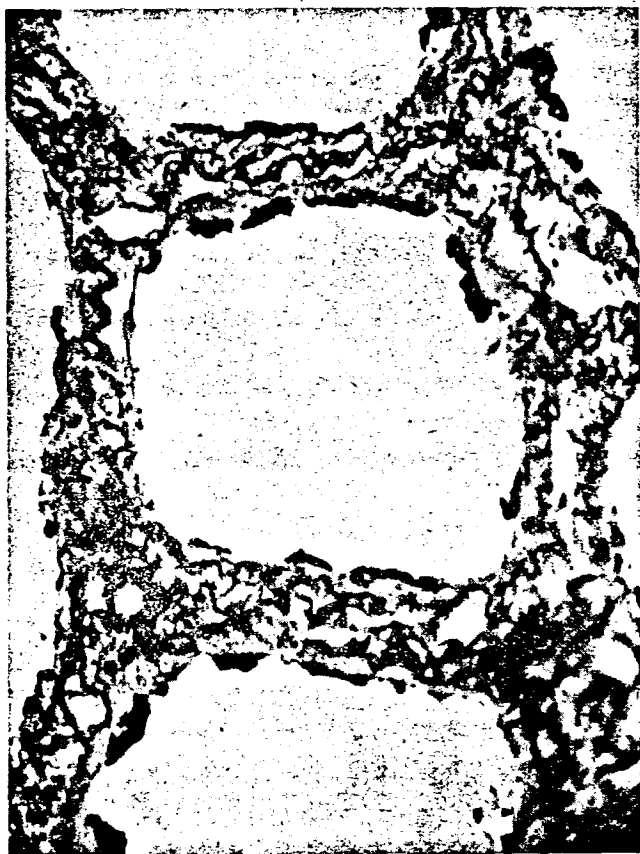


Fig. 3.—Twenty-five milliliters of saccharated iron oxide were put into the trachea an hour before this duck was killed. The iron appears as masses of black staining material on the epithelial surfaces of the parabronchi and in the interstitial tissue. Perl's stain for hemosiderin;  $\times 522$ .

RESPIRATORY TRACT OF DUCK



Fig. 4.—Either a capillary or lymphatic in the stroma near a bronchus showing an accumulation of iron staining material between and in the cytoplasm of the endothelial cells. The iron is passing from the bronchus into the circulatory system. Perl's stain for hemosiderin;  $\times 950$ .

*India Ink in Trachea of Ducks.*—Carbon particles were present macroscopically in the trachea of each of five ducks and in the lungs of three of the five birds given one injection of India ink suspended in liquid

petrolatum and killed 5 to 15 minutes later. Two of this group had carbon particles on the wall of the abdominal air sacs. No carbon particles were observed macroscopically in the respiratory tract of the five birds

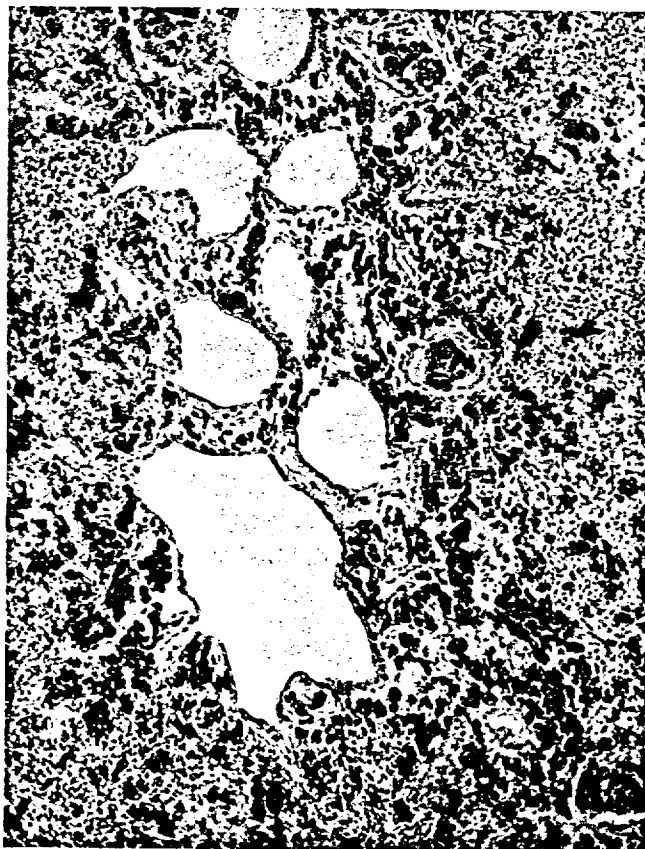
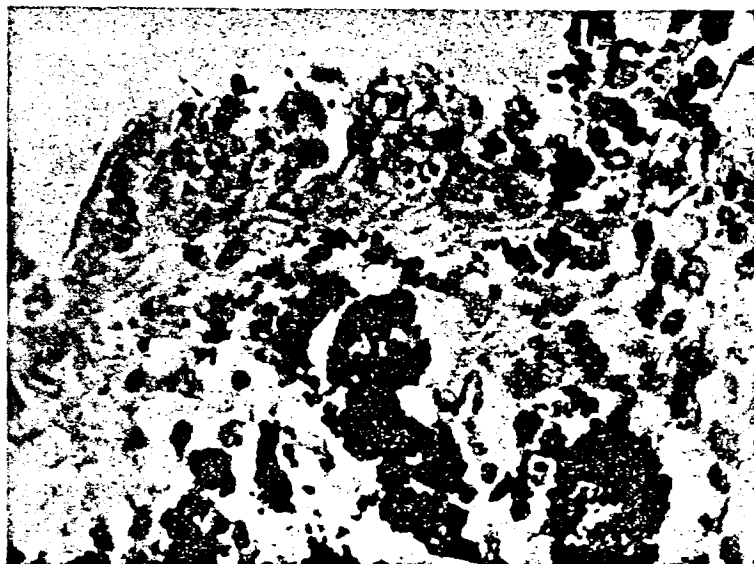


Fig. 5.—Twenty-five milliliters of India ink were put into the trachea five days before this duck was killed. The particles of ink are present in the stroma of the air capillaries and parabronchi. Hematoxylin and eosin stain;  $\times 150$ .

Fig. 6. — Particles of India ink are present between the epithelial cells that line this bronchus. Large masses of carbon particles have collected in the underlying stroma. Hematoxylin and eosin stain;  $\times 260$ .



given a single injection of the ink in liquid petrolatum and killed 48 hours later. Carbon particles were present macroscopically in the lungs of 4 of the 10 ducks similarly treated and killed 65 hours later.

Two ducks were given intratracheally 25 ml. of the suspension of India ink in distilled water. The birds were killed five days later. Particles of India ink were present in the lumen of the parabronchi and in the



Fig. 7.—Particles of India ink are present in the stroma adjacent to the parabronchi. Hematoxylin and eosin stain;  $\times 1,026$ .



Fig. 8.—Fibroblasts proliferate about the carbon particles in the stroma about a small bronchus. Hematoxylin and eosin stain;  $\times 360$ .

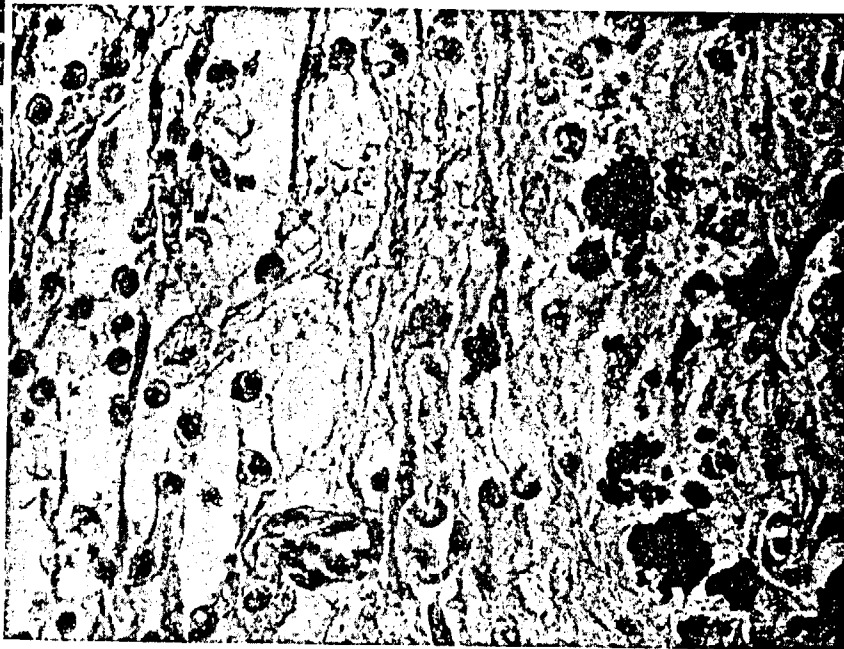


Fig. 9.—Macrophages phagocytize some of the particles of India ink while they are in the stroma. Hematoxylin and eosin stain;  $\times 665$ .



Fig. 10.—Carbon particles concentrated in the groups of lymphocytes. Local proliferation of lymphocytes occurs in the lung after the intratracheal injection of foreign material. Hematoxylin and eosin stain;  $\times 120$ .

lumen and the walls of the air capillaries (Fig. 5). In many areas, where only a few epithelial cells lined the respiratory tract, particles of India ink were present between the cells (Fig. 6). The particles of India ink, after passing through the layer of epithelium and into the adjacent stroma, formed varying-sized masses (Fig. 7). There was a proliferation of the fibroblasts in the stroma about some of the larger collections of particles of India ink (Fig. 8). Granules of India ink were present in the cytoplasm of some of the fibroblasts. Mononuclear cells, apparently histocytes, also were present in the stroma. Many of these cells phagocytized particles of India ink (Fig. 9). Particles of India ink were present within the group of lymphocytes throughout the lung (Fig. 10).

Ten ducks were given an intratracheal injection of the India ink suspended in liquid petrolatum daily for 10 days. They were killed 24 hours after the last injection. No carbon particles were observed macro-

scopically in the trachea. Carbon particles, however, were present macroscopically in the lungs of 2 and in the abdominal air sacs of 1 of the 10 ducks. Five of these ten birds did have macroscopic lipid material in the lungs and air sacs. Microscopic study of the respiratory tract of these 10 ducks showed the same distribution of the carbon particles as described above. In some of the larger collections of lymphocytes there was considerable carbon pigment. Extensive areas of acute and chronic inflammation were present in the lower portions of the lungs and sometimes also within the air sacs. A few polymorphonuclear leukocytes and lymphocytes infiltrated the mucosa of the trachea. Few granules of India ink were present in the wall of the trachea. Areas of inflammation occurred in the respiratory tract of ducks given intratracheal injections only of liquid petrolatum similar to that present in the birds given India ink suspended in liquid petrolatum.

## RESPIRATORY TRACT OF DUCK

*Liquid Petrolatum in Trachea of Ducks.*—An oily material was present macroscopically in the lungs and in the air sacs of 9 of the 10 ducks given daily injections of liquid petrolatum and killed 24 hours later. There was no macroscopic exudate in the trachea of any of these ducks; however, there was a minimal number of leukocytes and lymphocytes in the wall of the trachea in some of these birds. The lungs, in a majority of the ducks, showed an extensive acute and chronic reaction. A similar reaction was present in the wall of the air sacs.

One of the most interesting lesions observed in the lungs was a local proliferation of lymphocytes. Large groups of these cells were present in the wall of the larger bronchi, and smaller groups of similar cells were present in the stroma between the air capillaries. Sometimes these lymphocytes appeared to develop within the lumen of

small vessels, probably in lymph channels (Fig. 11).

Lipid material, as shown by the osmic acid stain, was present in the lumen of the bronchi, parabronchi, air capillaries, and air sacs. Sometimes small globules of lipid-staining material were present within the layer of epithelial cells that line the bronchi. After infiltrating the layer of epithelium that lines the bronchi, these lipid globules accumulated in the stroma in spaces that resembled lymphatics (Fig. 12). Many unidentifiable spaces, some of which may be capillaries, were filled with lipid-staining material. The lumina of some of the small blood vessels were partially filled with lipid material (Fig. 13).

*Lipodium Spores in Trachea of Ducks.*—Two ducks were given the suspension of Lipodium spores intratracheally and were killed 48 hours later. The lumina of many of the terminal bronchi, the parabronchi,



Fig. 11.—Foci of lymphocytes proliferate in the lungs after the intratracheal injection of foreign material. Hematoxylin and eosin stain;  $\times 380$ .



Fig. 12.—Lipid staining material in the lumen of either lymphatics or blood vessels in the interstitial tissue of the lung after the intra-tracheal injection of liquid petrolatum. Osmic acid stain;  $\times 190$ .

and the air capillaries were filled with these spores. Sometimes the spores appeared to have adhered to the surface of the epithelium lining the air passages, while in other

areas the spores passed through the layer of epithelium and were present in the adjacent stroma (Fig. 14). Sometimes giant cells surrounded these spores (Fig. 15).

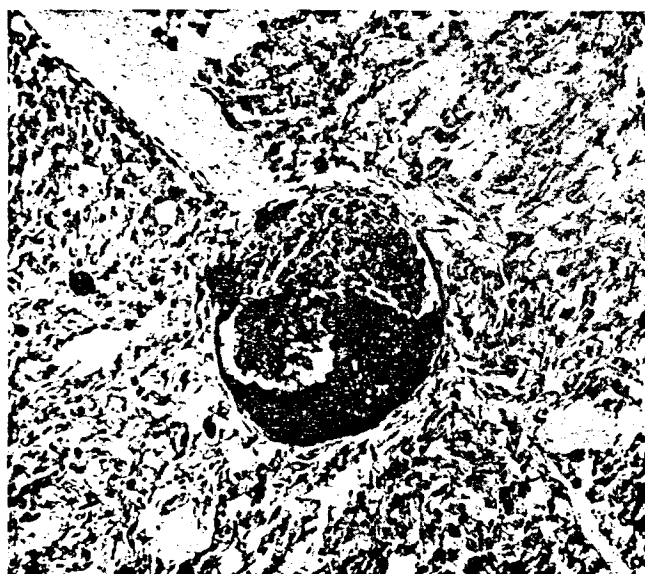


Fig. 13.—The lumen of a small blood vessel in the lung filled with lipid staining material. Liquid petrolatum was put into the trachea 63 hours before this duck was killed. Osmic acid stain;  $\times 280$ .



Fig. 14.—A spore in the wall of a bronchus 48 hours after the intratracheal injection of an aqueous suspension of *Lipodium* spores. Hematoxylin and eosin stain;  $\times 260$ .

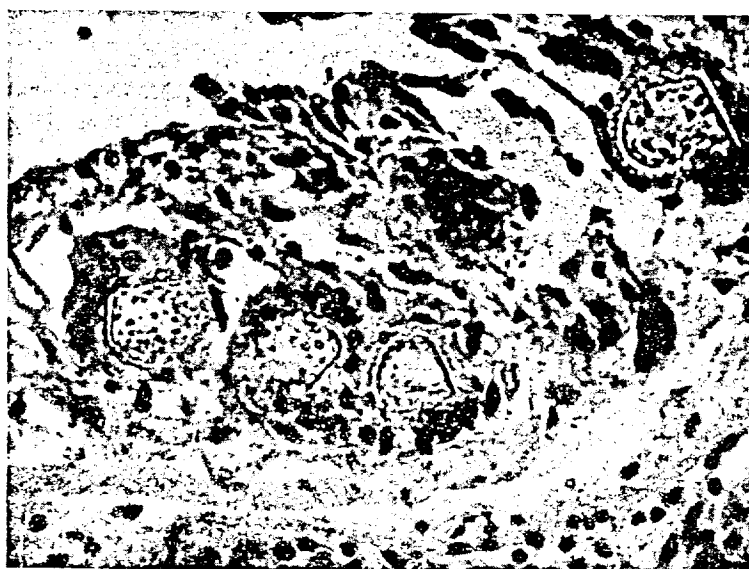


Fig. 15.—*Lipodium* spores in the stroma beneath the epithelium lining a bronchus. These spores are surrounded by giant cells. Hematoxylin and eosin stain;  $\times 760$ .

Apparently the spores injured the lining epithelium as they passed from the lumen of the bronchi through the layer of epithelium into the underlying stroma.

#### Comment

Fluorescein sodium is rapidly transferred from the respiratory tract of the duck into the peripheral circulation. A fluorescent material was present in the blood removed from a leg vein one minute after 50 ml. of 0.5% solution of fluorescein sodium was put into the trachea. It would seem that such a liquid passes rapidly through the layer of epithelium lining the lower portion

of the respiratory tract to reach the adjacent stroma. Here it diffuses through the wall of blood vessels and/or lymphatics. The sections removed from the respiratory tract after the intratracheal injection of saccharated iron oxide and stained by the Perl technique for hemosiderin show a large amount of blue-staining material in the stroma and in the wall of some of the smaller vascular channels.

The osmic acid stains made from the tissues of the respiratory tract of ducks given liquid petrolatum intratracheally have many small lipid globules in the layer of epithelium lining the smaller bronchi, the

Rigdon

117/225

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parabronchi, and the air sacs. In the stroma immediately adjacent to these epithelial structures are large masses of lipid-staining material. Usually it is impossible to say whether these collections of fat are in a lymphatic or a capillary or are free in the tissue spaces. In a few sections small blood vessels did have lipid-staining material within their lumina. The presence of lipid-staining material within the lumen of these vessels would suggest that globules of the liquid petrolatum had entered either a lymphatic that communicated with a blood vessel or had entered directly a blood vessel.

Sections of the respiratory tract from ducks given intratracheal injections of India ink show very nicely that particulate material passes directly through the layer of epithelium lining the air passages to reach the adjacent stroma. Many of the carbon particles remain in the stroma; however, some are phagocytized. Lipodium spores also pass directly through the layer of epithelium lining the respiratory tract in a manner similar to the particles of India ink and the globules of liquid petrolatum.

This study shows that fluids and particulate material leave the respiratory tract by passing between the epithelial cells that line the walls. After reaching the stroma, some of the particles enter the lumen of lymphatics and/or blood vessels by passing between the endothelial cells that form their walls. Some of the particles, while in the stroma, are phagocytized by macrophages. No doubt, these phagocytic cells with particulate material subsequently enter the pulmonary lymphatics and ultimately reach the lumen of blood vessels.

Particulate material in mammals, as is well known, is phagocytized usually within the pulmonary alveolae. These phagocytic cells then enter the pulmonary lymphatics and are filtered out by the regional lymph nodes. In the duck, phagocytosis has not been observed to occur within the respiratory tract. Small particles are phagocytized by macrophages and fibroblasts within the stroma after they have passed the layer of

epithelium that lines the air passages. Giant cells surround the larger particles, such as Lipodium spores, in an attempt to phagocytize them. It would seem from this study that much of the particulate material in the respiratory tract of the duck enters the lymphatics and the vascular system directly without first being phagocytized. Some of the foreign particles are retained within the collections of lymphoid cells that develop in the lungs and in the wall of the bronchi.

The air sacs in the duck appear to be the principal area where particulate material is removed from the respiratory tract. These spaces are lined by a single layer of cuboidal or low columnar epithelium. Particulate material, of course, passes such an epithelial barrier more readily than it passes through the layer of stratified epithelium that lines the trachea.

The mechanism of the removal of fluids and particulate material from the respiratory tract of the duck is most interesting when one compares it with the general concept of the local changes that occur in tissues in inflammation. The latter mechanism has been summarized recently by Gozsy and Kato<sup>13</sup> as follows:

This response is manifested by a suddenly acquired absorptive capacity of the capillary endothelial cells and the whole inner surface of the capillary tube, providing the endothelial cells with a storing and phagocytizing activity. This phenomenon is followed by a progressively increasing permeability of the cement substance, permitting the active transport of damaging particulate matter, previously absorbed on the vascular endothelium, into the perivascular space.

In the removal of fluid and particulate material from the respiratory tract of the duck, the particles pass through the layer of epithelium into the loose stroma where there are lymphatics and capillaries. The particles then pass from the stroma between the endothelial cells into the vascular and/or lymph channels. In this process some of the particles appear to be phagocytized by the endothelial cells that line the channels. Although we have no experimental data to support the suggestion that these phagocytized particles ultimately are released into

## RESPIRATORY TRACT OF DUCK

the circulating lymph or blood, it would seem most likely that such does occur. The removal of particulate material from the respiratory tract of the duck is essentially the reverse to that which occurs in inflammation. In the latter, colloidal dyes, antibodies, and India ink pass from the lumen of the capillaries into the adjacent stroma, while particles pass from the stroma into the capillaries during removal from the respiratory tract. The local factors that make possible this transfer of particles from the respiratory tract to the circulatory system need additional study.

### Summary

In the duck, saccharated iron oxide and fluorescein sodium, when put into the respiratory tract through the trachea, pass directly through the layer of epithelium lining the respiratory tract into the stroma and through the wall of the blood vessels and/or lymphatics. Particles of India ink, globules of petrolatum, and Lipodium spores, when put into the respiratory tract of ducks, migrate through the layer of epithelium and either enter the blood vessels and/or lymphatics directly or are phagocytized within the stroma by fibroblasts and histocytes. The transfer of fluids and particulate materials from the respiratory tract of the duck to the vascular system occurs primarily in the air sacs. The variation in the mechanism of removal of particulate material in the duck and in mammals no doubt is necessitated by the differences in the respiratory and lymphatic systems. There are no lymph nodes in the duck as there are in mammals.

Department of Pathology, University of Texas—Medical Branch.

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## Keratoacanthoma

### *Experimentally Induced with Methylcholanthrene in the Chicken*

R. H. RIGDON, M.D., Galveston, Texas

In 1934, Ferguson Smith<sup>1</sup> described a self-healing squamous-cell epithelioma in the skin of a man 23 years of age. In 1955, Ereaux and associates<sup>2</sup> reported a case in which there were widespread self-healing epithelial tumors in the skin of a white man 41 years of age that were considered keratoacanthomas. The clinical and histologic characteristics of this lesion have been described.<sup>2-18</sup> A new classification of keratoacanthomas in three histological types, Types 1, 2, and 3, has been suggested recently by Ghadially.<sup>19</sup>

In considering the etiology of the localized type of keratoacanthoma, Anderson,<sup>2</sup> in the discussion of Ereaux's paper, says, "One is impressed . . . as to the frequent history of preceding trauma, such as a cut, scratch, or the squeezing of a blackhead. There is no doubt that very similar, if not identical, lesions occur in those exposed to tar." Actinic or oil sensitization has been implicated,<sup>16</sup> also sunlight<sup>18</sup> and oil and tar.<sup>4</sup> There are observations that would suggest a viral origin.<sup>2</sup> Since some of the cases have been observed in members of a family, there may be a genetic factor.<sup>3,17</sup>

Submitted for publication July 29, 1958.

Professor of Pathology, The University of Texas—Medical Branch.

This study was aided by research Grants C-1469 (C-5) from the National Cancer Institute of the National Institutes of Health, Public Health Service, and from the Tobacco Industry Research Committee.

In the study of carcinogenesis in the chicken,<sup>20-22</sup> we have observed lesions in the skin following local applications of methylcholanthrene characterized by a macule that subsequently progressed in size and ulcerated. Histologically, the lesion has all the characteristics of a squamous-cell carcinoma, but it spontaneously regresses. This lesion in the chicken is described at this time because of its similarity to keratoacanthoma.

### Methods and Material

The technique used to produce these tumors has been reported.<sup>20</sup> A 0.25% solution of methylcholanthrene in acetone was applied to the skin of the body beneath the wing and to the undersurface of the wing. The acetone solution of methylcholanthrene, 0.2 ml., was used on the day-old chicks, and 1 ml. was used on the older birds. The applications of the carcinogen were made daily, except for Saturdays and Sundays. The total number of applications for any one bird varied from 1 to 30. In some experiments the feathers were plucked, either before, during, or after the methylcholanthrene was applied. The lesions sometimes were either biopsied or completely excised for histologic study.

### Results

The details of the experimental observations made in this study have been reported.<sup>20,21</sup> The earliest tumor occurred 23 days after the first application of methylcholanthrene. The longest interval between the first application of methylcholanthrene and the development of a tumor was 128 days. Eight was the largest number of

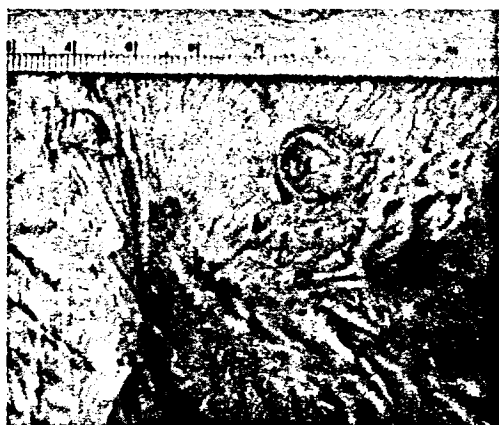


Fig. 1.—The center of these squamous-cell tumors is filled with keratin. Hematoxylin and eosin; reduced 20% from mag.  $\times 19$ .

tumors observed at one time in any one chicken. The lesions, when first observed, were nodules about 2 mm. in diameter. They progressively grew, and their centers were filled with keratin (Fig. 1). They subsequently ulcerated (Figs. 2, 3, and 4). The size of the ulcers varied from 3 to 12 mm. The base was granular; the periphery was hyperplastic, and the edges were undetermined. Healing seemed to progress rapidly from both the base and the periphery; a typical scar resulted.

Histologically, the lesion always was characterized by squamous cells (Fig. 5). Intercellular bridges (Fig. 6) were conspicuous in some, while in others typical

epithelial pearls were present. Keratin and mitotic cells were numerous in some of the lesions (Figs. 7 and 8). Polymorphonuclear leukocytes were present in the crater of the tumors. Leukocytes, mononuclear cells, and groups of lymphocytes were present in the surrounding dermis (Figs. 9 and 10). Polymorphonuclear leukocytes frequently were prominent about the periphery of the tumor. Extensive degeneration occurred in the squamous cells at the periphery of the growth (Fig. 11). The degree of degeneration varied in the different lesions, both within the same chickens and in different birds. Ultimately, the squamous cells in the tumor completely degenerated; the leukocytes phagocytized the debris, and collagen fibers filled the area.

This degenerative change in the tumor is most pronounced and seems to begin at the periphery of the growth. It proceeds inward until all the epithelial cells are destroyed. The time for regression varied from two weeks to two months.

#### Comment

The tumors that develop in the skin of chickens following the local application of methylcholanthrene resemble in many ways the keratoacanthoma described in man by McNulty and Sommers.<sup>16</sup> They have described this lesion as follows:

Fig. 2.—This chicken was 77 days of age when 1.0 ml. of the solution of methylcholanthrene was applied daily for 30 days except for Saturdays and Sundays. The lesion was first observed on the 75th day after the first application of methylcholanthrene. Photographed on the 99th day.



# KERATOACANTHOMA

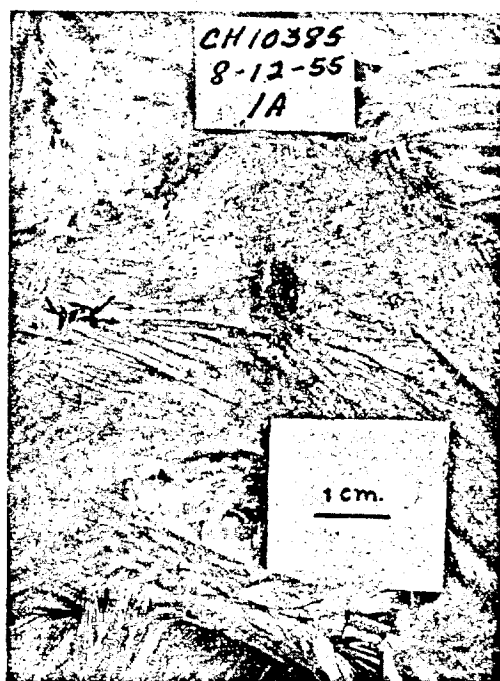


Fig. 3.—Treated same as chicken shown in Figure 1. Photographed on the 105th day after the first application of methylcholanthrene.



Fig. 4.—Treated same as chicken shown in Figure 1. Photographed on the 47th day after the first application of methylcholanthrene. The tumor was 1.0 cm. at this time.

... crateriform, with masses of keratin packed in centrally and superficially . . . On section the mass is pulpy, moist, keratinaceous, exudes a cloudy fluid, and has a crumbling yellow-brown appearance . . . rounded masses of partly keratinized cells with abundant ground-glass-like eosinophilic cytoplasm and prominent intercellular bridges are bulging downward into the dermis and everting the

marginal unaffected epithelium . . . . The basement membrane boundary zones are obscured or lost, and a heavy infiltrate of leucocytes surrounds and overflows into the epithelial downgrowths . . . . Under higher magnification the diffuse mingling of leucocytes, particularly neutrophils, eosinophils, and lymphocytes, with sheets of epithelium is further emphasized . . . . Plasma cells, lymphocytes

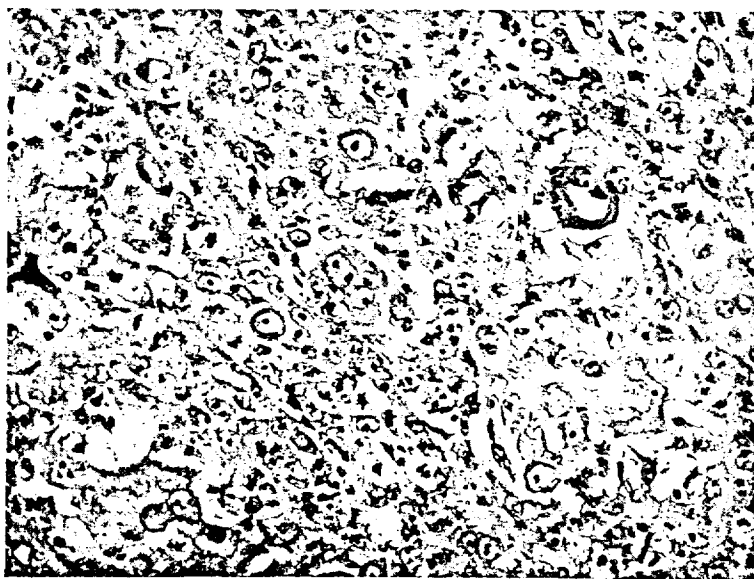


Fig. 5.—The squamous epithelial cells vary in size and shape. Epithelial pearls do occur. Mitotic figures are present. Hematoxylin and eosin; reduced 10% from mag.  $\times 340$ .

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Fig. 6.—Intercellular bridges are present in some of these squamous-cell tumors in the chicken. Hematoxylin and eosin;  $\times 800$ .



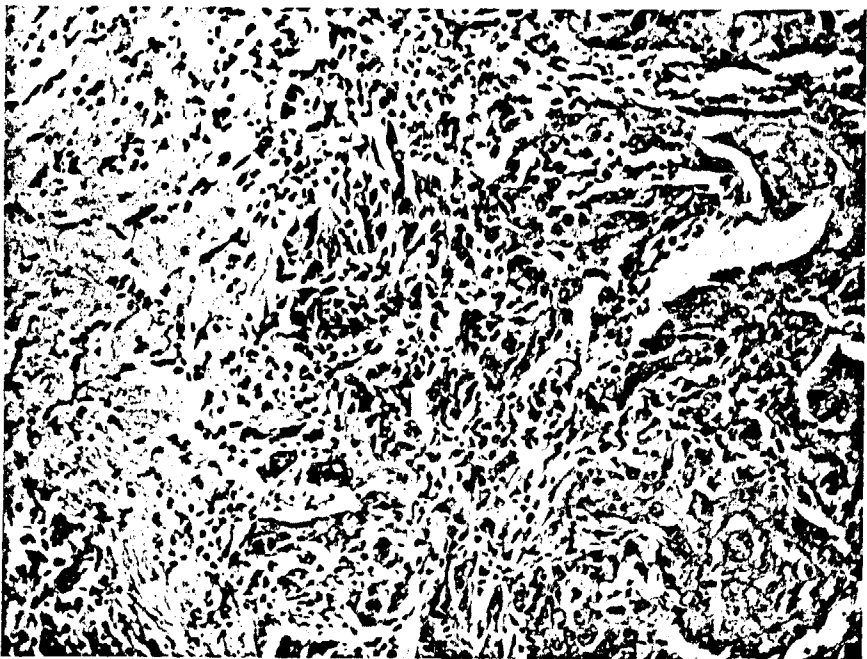
Fig. 7.—These are groups of squamous cells with ground-glass-like eosinophilic cytoplasm in focal areas of these tumors. Observe the large number of polymorphonuclear leukocytes. Hematoxylin and eosin;  $\times 340$ .

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Fig. 8.—Atypical mitotic cells  
are present in these tumors. He-  
matoxylin and eosin;  $\times 800$ .



Fig. 9.—The periphery of these  
tumors is usually infiltrated with  
lymphocytes and leukocytes. He-  
matoxylin and eosin;  $\times 170$ .



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Fig. 10.—Many lymphocytes usually are present about the periphery of these lesions. Hematoxylin and eosin;  $\times 340$ .



Fig. 11.—The nuclei of the squamous cells at the periphery of these regressing lesions are pyknotic. Subsequently, these cells atrophy and ultimately completely disappear. Hematoxylin and eosin;  $\times 340$ .

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## KERATOACANTHOMA

and macrophages are around the epithelium in abundance. Degenerative cytologic epithelial changes are uniformly present and distinctive . . . Mitoses are abundant in the hyperplastic epithelium and abnormal mitotic figures with chromosomal fragmentation and dispersion, usually outnumber the normal mitoses . . . In the adjacent dermis, detached epithelial cells heavily infiltrated with leucocytes are found in keratoacanthoma.

The similarity of this lesion induced with methylcholanthrene in the skin of the chicken and the description of keratoacanthoma, as given by McNulty and Sommers,<sup>16</sup> is obvious. It is my opinion, and the opinion of others who have studied this lesion in the skin of chickens, that it is histologically a low-grade malignant epithelial tumor. However, since it regresses and does not metastasize, it has been described as a "squamous-cell carcinomatoid tumor,"<sup>21</sup> although originally we reported it as a "squamous-cell carcinoma."<sup>20</sup> The problems in the differentiation of keratoacanthoma, squamous-cell carcinoma, and pseudoepitheliomatous hyperplasia are readily appreciated and have been discussed frequently.

The most interesting features about keratoacanthoma in man and the carcinomatoid tumors in chickens induced by methylcholanthrene are their histologic resemblance to squamous-cell carcinomas and their spontaneous regression. An increasing number of malignant tumors that regress spontaneously is being found in man and animals.<sup>23-27</sup> Little is known, however, as to the mechanism of regression. Haaland<sup>28</sup> suggested that a change must occur within the tumor cell to explain this phenomenon. Huggins<sup>29</sup> has emphasized the role of hormones in the regression of certain neoplasms. Cowdry,<sup>30</sup> in discussing the problems of "unexplained disappearance and alterations in the malignancy of cancer cells," suggests that "spontaneous modifications in the volume, or growth, of a cancer could result from many variables both of the malignant cells and of the stroma." Experimentally induced hemangiomas spontaneously regress as a result of a mechanical obstruction that occurs within the vascular channels. With the progressive decrease in

blood flow through the vascular channels, tissue anoxia may become the significant factor in the mechanism of this spontaneous regression.<sup>31</sup>

The observation that the degree of cellular degeneration is more marked in the cells at the periphery of the chicken tumor induced by methylcholanthrene would suggest that some cellular injurious factor reaches these cells by way of the tissue fluid. Antibodies formed elsewhere in the body, reacting with the antigen represented by the proliferated epithelial cells, could account for this degeneration of the tumor cells. The degeneration might be expected to begin at the periphery of the lesion. In support of this hypothesis are the observations of Pollard and Bussell,<sup>32,33</sup> who found an inhibition of growth of methylcholanthrene-induced tumor tissue when the transplants were grown in the presence of splenic tissue obtained from mice previously inoculated subcutaneously with methylcholanthrene. This tumor-destructive effect could not be demonstrated with "normal" spleens or with the spleens from mice supplying the tumor tissue. Furthermore, this reaction could not be induced with spleens from mice treated with other carcinogens; too, when a carcinogenically induced transmissible tumor involuted, the recovered host was refractory to reinoculation with the same tumor.

It would seem of interest to observe persons with keratoacanthomas closely from an immunological standpoint. A regression of these lesions may be related in some way to an immune process. In view of the histological similarity of the keratoacanthoma and a low-grade squamous-cell carcinoma, it might be that some immune process occurs in the person to account for the regression of the former lesion. A history referable to the resistance to bacterial infections, allergic manifestations, and serologic and chemical observations on the serum might contribute something to the mechanism of regression of neoplasms, a process that rarely occurs in man but does occur frequently in laboratory animals.

Ghadiaily,<sup>19</sup> in 1958, pointed out the similarity of lesions produced by 9:10-dimethyl-1:2-benzanthracene in the skin of the rabbit to keratoacanthomas in man. The paper by Ghadiaily<sup>19</sup> came to my attention after this manuscript had been prepared. It is of considerable interest, however, to observe the close similarity of the history and the morphology of these lesions in man and the rabbit. Too, the lesions in the chicken are very similar to those in the rabbit. Keratoacanthomas, according to Ghadiaily,<sup>19</sup> appear to start by hyperplasia of the hair follicles and metaplasia of the sebaceous glands. Fragments of hair shafts often persist; this lends support to the suggestion that these lesions arise from hair follicles and not as downgrowths from the epidermis.<sup>19</sup> The fact that multiple lesions histologically similar to keratoacanthomas can be produced in the rabbit with carcinogens suggests the possibility that cases of multiple self-healing carcinomas may be no more than multiple keratoacanthomas arising in a genetically susceptible group of persons exposed perhaps to an undetected carcinogenic stimulus.<sup>19</sup>

### Summary

A squamous-cell tumor occurs in the skin of chickens after the local application of methylcholanthrene. This tumor is characterized by a crater in which there is a large amount of keratin and leukocytes. Lymphocytes, histocytes, and leukocytes are present at the periphery of the growth. The tumor spontaneously regresses. The similarity of this squamous-cell tumor in the chicken to the keratoacanthoma in man is discussed.

University of Texas Medical Branch.

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Vol. 79, Feb., 1959

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CONFIDENTIAL

October 8, 1958

Robert C. Hockett, Associate Scientific Director  
Tobacco Industry Research Committee  
150 East Forty Second Street  
New York 17, N.Y.

Dear Mr. Hockett:

We are moving slowly with our experimental study of tobacco tar in the respiratory tract of white Pekin ducks. We have not found any tumors in the respiratory tract of any of our birds. I have not been impressed with the inflammatory reaction in the lining. There has occurred one significant point in this study. We had three ducks that were kept for eighteen months that showed cirrhosis of the liver. This cirrhosis is the same type of change that we have previously reported following the local application of methylcholanthrene to the skin of ducks. We are now completing some studies in which methylcholanthrene was put into the trachea and the birds developed cirrhosis of the liver. Ducks also developed cirrhosis of the liver following oral administration of methylcholanthrene.

We don't have but a few ducks given a tobacco tar and observed for a long period of time. Such an experiment is tedious and requires much time. I question whether it would be worthwhile to repeat except for the possibility of this liver lesion.

You may be interested in knowing that we have completed and had accepted for publication an article discussing the normal respiratory tract in the white Pekin duck. This was made possible by support from your group. This paper was accepted by Poultry Science in June, 1958, and is entitled "The Respiratory System in the Normal White Pekin Duck."

We are completing a second study aided by the Tobacco Industry Research Committee in which we discuss the effect of methylcholanthrene on the respiratory tract. I hope that this work will be completed in three or four months.

We are now able to check some of the tissues with the new fluorescent microscope that we obtained through money supplied to us by your organization. We are trying to learn how to use this instrument and feel that it will be of help to us in this overall problem.

I wanted to let you know the general outline of what we are doing and this is why I have taken this opportunity to write you the above. With kind regards,

Yours truly,

/s./

R.H. Rigdon, M.D.  
Professor of Pathology

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# CANCER OF THE LUNG FROM 1900 TO 1930

R. H. RIGDON, M.D., and HELEN KIRCHOFF, Galveston, Texas

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## CANCER OF THE LUNG FROM 1900 TO 1930

R. H. RIGDON, M.D., and HELEN KIRCHOFF, Galveston, Texas

INTEREST IN CANCER of the lung began in France with the publication, in 1810, of Bayle's article (10) and rapidly spread to England and Germany. Scientific interest continued to progress, as indicated by the number of publications, and by the beginning of the twentieth century it had already spread to many European countries and to the Americas. Between 1900 and 1930 many theses and dissertations on this subject were submitted to various faculties of medicine to fulfill academic requirements for advanced degrees. Reviews and monographs were beginning to appear and in 1928 an international symposium was held in London for a discussion of many problems referable to cancer of the lung. These discussions included the frequency, etiology, histogenesis, pathology, clinical diagnosis, and treatment. It would be of interest for all of us today to read the number of excellent contributions made by many investigators throughout the world between 1900 and 1930, and to know that men were just as interested at that time to establish the etiology, and to account for the increase in frequency, of cancer of the lung as we in the United States have been during the last few years.

From the Department of Pathology, University of Texas, Medical Branch.

This is part of a co-operative study with the Medical College of Virginia.

There can be only one thing accomplished by a review of the literature on cancer of the lung at this particular time and that is to collect and briefly emphasize some of the data published between 1900 and 1930. It seems to us that the inscription engraved in stone on each side of the north entrance of the Archives Building in Washington, D.C. is apropos to this problem of cancer of the lung: "What is past is prologue. Study the past."

Pepper (116), writing in 1850, said, "It is not until quite recently that this subject (of pulmonary cancer) has fully engaged the attention of the profession; hitherto, such cases were viewed as mere matters of medical curiosity 'not known to be in any degree influenced by medicine, and too rare to be of much practical importance.' There is good reason to believe, however, this disease is of much more frequent occurrence than is commonly supposed, and that in a vast majority of cases it entirely escapes detection, owing to the great difficulty which attends its diagnosis." Reinhard (126) found 27 published cases in 1878. Werner (168), in 1891, found 9 cases fully verified. Wolf (171), in 1895, reported 31 cases which had been diagnosed since 1885, and Pässler (115), in 1896, found 70 and added 4 of his own. Boecker (16), in 1910, estimated that about 100 cases could be found in the literature. Adler (2), in 1912, collected 374

cases of pulmonary carcinoma and 94 cases of pulmonary sarcoma. Weller (164), in 1913, collected 89 cases that he considered authentic primary carcinomas of the larger bronchi and investigated the pathological and clinical features of the group. In 1918, McMahon and Carman (102) found in the literature 428 cases which they considered to be authentic.

Difficulties and errors in the clinical diagnosis of lung cancer have been recognized since Bayle (10), in 1810, reported his group of 3 cases. Pässler (115), in 1896, stated, "Actually from this abundance of material only a fraction can be used for our information on primary carcinoma of the lungs. First, because no cases without a post-mortem report can be considered and secondly, as has been emphasized already by some authors, those cases also have to be eliminated whose carcinomatous nature . . . cannot be considered as positive." Incredible as it may be, one may read in Sehart's work, published in 1904, that of 178 cases of lung cancer a correct clinical diagnosis was made in only 6 (75). This is some improvement, however, over the 100 per cent wrong diagnoses observed by Jeuther *et al.* (70) between 1894 and 1899. Sweany (153), after reviewing the problem of clinical diagnosis of lung cancer, concluded that at the beginning of this century the percentage of correct diagnoses was 5 per cent. In 1912, Adler (2) had this to say, "Is it not somewhat humiliating to realize that the difficulties of diagnosis are still so great as to prevent the best and most experienced medical men, with all the advantages of a large hospital, from discovering almost one-fifth of all carcinomata that come before them? . . . The physician must be imbued with the conviction that malignant pulmonary disease occurs much more frequently than is commonly believed and that he may meet it any day in his practice among the young, as well as among the old."

Difficulties in the diagnoses of lung cancer have continued to be recognized. It was pointed out by Adler (2) that "even the diagnoses made on the autopsy table are not always reliable. . . . It may happen also that the most careful and searching autopsy will not furnish the true diagnosis until a thorough microscopical examination has been made." Weller (164), after reviewing a group of 89 cases, found that the correct clinical diagnosis had been made in only 10 and suggested "with the additional aids now available the proportion of diagnosed cases should be very much

increased." Wells (166) found a diagnostic error of 36.5 per cent in a group of 578 cases of cancer studied in 1923, and he said, "Such a high ratio of incorrect diagnoses in a great hospital might seem to be evidence of something wrong with the hospital, but we find that other institutions dealing with a similar class of cases, in which most of the cancers coming to necropsy are of the internal organs, exhibit not dissimilar figures."

The clinical diagnosis of pulmonary cancer began to improve about 1915, although Scott and Forman (140), in 1916, found one correctly diagnosed case in their series of 4. In 1921, Lubarsch (92) found a correct diagnosis had been made in 240 of 458 cases, or 52 per cent. Barron (8), in 1922, approached the problem of the diagnosis of lung cancer in a different manner from that of others; he said, "Undiagnosability simply resolves itself into unfamiliarity with the more or less characteristic signs and symptoms." Considerable attention was given in 1927 by Probst (124), Wahl (160), and Katz (73) to this problem of the diagnosis of lung cancer. Probst (124) found that the correct clinical diagnosis had been made in 36.9 per cent of the 65 cases he reviewed and he deplored this low percentage "in spite of systemically applied x-ray examination." He cited the observation that "according to Bilz, Lubarsch, Sachs, Seyfarth, and others the diagnosis is made correctly in about 50 per cent." Sweany (153) considered the correct percentage diagnosis "had barely risen to 47 per cent" in 1925. Fried (40), in 1925, found only 2 cases in a group of 10 that were correctly diagnosed antemortem at the Peter Bent Brigham Hospital in Boston. Physicians in 1926 and 1930 were being warned to include carcinoma of the lung in their differential diagnoses (38, 39). Although by 1926 there were the additional aids of x-rays and the bronchoscope for the diagnosis of lung cancer, Fishberg (38) pointed out that a careful history and physical examination were of much greater importance than x-rays, particularly in the recognition of early cases.

It would seem to me that by 1930 there had occurred a marked improvement in the clinical diagnosis of pulmonary cancer. We would estimate this to be about 50 per cent in the practice of the better physicians, clinics, and hospitals. Obviously the percentage of correct diagnosis would be progressively lower in the practice of

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those who had less training and who did not have access to adequate diagnostic facilities. This fact becomes a most important one when attempting to establish the frequency of lung cancer between 1900 and 1930. Adler (2), commenting on this problem in 1912, said, "The death certificates on which burial permits are officially given are often ludicrously insufficient. For this reason the United States Census is entirely useless for our purposes . . . . It is therefore impossible to say, from the figures given by the United States Census concerning causes of death, how many persons mentioned as having died from tuberculosis, pneumonia or kindred diseases, may not really have died from lung tumors." This skepticism in America regarding the value of vital statistics for establishing the frequency of tumors likewise was expressed by Weller (163), Professor of Pathology at the University of Michigan, and by Wells (166), Professor of Pathology at the University of Chicago.

Hoffman (58), in 1929, pointed out that the Census Office in 1914 made the first attempt to present a comprehensive statement on cancer. In that year the death rate for cancer of the lung was 0.6 per 100,000 of the population and in 1924 it had increased to 1.6. Obviously this was a tremendous increase in a period of only 10 years. A most conspicuous feature of the statistics, as reported by Hoffman, was the variation in the frequency of cancer of the lung in the United States and in Canada. Between 1919 and 1923 the rate for Albany, New York was 2.5 and for the white population of New Orleans it was 2.8. From 1920 to 1924 the rate for San Francisco was 4.7, Boston 3.9, Province of British Columbia 2.1, Province of Saskatchewan 0.5, and for the city of Winnipeg 3.3.

Gilliam (45), Chief of the Epidemiology Section of the National Cancer Institute, recently pointed out that "the International Lists of Causes of Death did not separately list cancer of the lung and pleura, or even cancer of the respiratory system as a whole, until the Fourth Revision—first used in 1930. . . . Prior to 1930 the term bronchogenic carcinoma was lumped with 90 other cancers of various sites. Between 1930 and 1938, when this diagnosis appeared on a death certificate, it was tabulated with cancer and other malignant tumors of other respiratory organs (47C), that is, with tumors other than those designated as larynx (47A) or as lungs and pleura (47B). It was not until the Fifth Revision

came into use in 1939 that deaths which physicians charged to bronchogenic carcinoma were separately identified in official statistics."

All the literature supports the opinion that cancer of the lung has progressively increased since 1810. Gilliam (45) stated that "the rate of increase in recorded mortality was greatest in this country (America) between 1914 and 1930 and that it has been declining since." It was so common in 1927 that Weller (163) referred to it figuratively as an "epidemic." During the period from 1900 to 1930 a tremendous number of statistical studies based upon autopsies was made in France (62, 89, 106), Germany (15, 21, 22, 72, 73, 76, 92, 125, 160, 169), Russia (86, 112, 117, 121), England (19, 30, 33, 65, 104, 114, 145), Holland (31), Austria (36, 97), Italy (29, 158), Brazil (96), Switzerland (124), Czechoslovakia (99), Spain (28), Canada (20, 57, 80), Hungary (172), Denmark (64), Argentina (152), and America (8, 18, 40, 46, 47, 50, 90, 94, 131, 163, 165).

Excellent reviews on the increase of pulmonary cancer were included in the papers by Wahl (160), Probst (124), Katz (73), Weller (163), Barron (8), Moise (107), and Brunn (26). Probst (124) compiled data from 24 statistical studies made in Europe. After the elimination of those with incomplete figures, there were 88,750 autopsies which were performed between 1900 and 1925; in this particular group of autopsies 4.3 per cent of all cancers were pulmonary. In 30,468 autopsies performed between 1892 and 1931 by Americans, 5.5 per cent of all cancers were primary in the lung (40, 43, 71, 81, 94, 100, 107, 131, 149, 150, 163).

Between 1900 and 1930 there was one point that many investigators often wished to establish, and that was, "Is the increase in cancer of the lung real or only apparent?" Adler (2), in 1912, expressed the opinion that "there seems hardly room for doubt that the increase in the percentage of lung tumors is to be attributed mainly to the increased attention paid to these types of tumors and the greater care and more extensive microscopic investigation with which autopsies are carried out at present." Barron (8), in 1922, concluded that "not only is there an absolute increase in the number of cases but there is a three fold relative increase." Probst (124), in 1927, thought that the increase might be only apparent and due to more accurate and finer diagnosis. Huguenin (62), in 1928, said,

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#### 4 *International Abstracts of Surgery* · August 1958

"We cannot form an opinion on the relative frequency; we can only affirm that primary cancer in the lung is not rare." Schall (135), in 1928, felt that it remained an open question whether there had been a true or an apparent increase due to more accurate diagnosis. Homann (59), in 1930, reviewed the available literature, discussed the alleged increase in frequency of lung cancer during recent years, and concluded that the increase was apparent and not real. This increase, according to Homann, may be influenced by the bronchoscopic, roentgenographic and histologic techniques. Von Glahn (46), in 1930, said the available statistics left him "in doubt as to whether there has been an actual increase of lung cancer."

Cancer of the lung in 1905 occupied fifth place among malignant tumors and in 1925 it had reached second place (156). In the region of Trieste, Italy, Ferrari (37) observed that before 1910 cancer of the lung occupied eighth place, fourth place between 1911 and 1920, and by 1921 to 1926 it was in second place. Barron (8), in 1922, considered carcinoma of the lung sixth among all malignant conditions, while in 1930, according to Rosahn (131), it was in fifth place. Cancers of the lung and bronchi were found to be second in frequency to cancer of the stomach in a group of 22,139 cases collected between 1925 and 1933 (32). This progressive increase in cancer of the lung is interesting in view of what King and Newsholme (79) pointed out in 1893: "The increase in cancer is only apparent and not real, and is due to improvement in diagnosis and more careful certification of the causes of death. This is shown by the fact that the whole of the increase has taken place in inaccessible cancer difficult of diagnosis, while accessible cancer easily diagnosed has remained practically stationary."

In 1930 we find unanimity in the increase of cancer of the lung; however, a division did exist as to whether this increase was real or only apparent. Hamman (51), in discussing the confusion referable to the statistics on cancer of the lung, said that there were not sufficient data to settle the dispute whether the increase was real or apparent, and noted that "two authors, Rosahn and Fried, using the same statistical material, arrived at opposite conclusions." Rosahn wrote that the postmortem incidence of primary carcinoma of the lung was steadily increasing and this increase was real and absolute,

while Fried stated that the increase was very likely more apparent than real.

It has been pointed out in many of the statistics that cancer of the lung occurred more frequently in men from 50 to 70 years of age (62, 63, 76, 115, 124, 160). Karrenstein (72), in 1908, found the average age of patients with cancer of the lung to be 55.4 years. Weller (164), in 1913, observed that most of his cases occurred between the ages of 56 and 60 years. Brunn (26), in 1926, found 90 per cent of his cases occurring between the ages of 40 and 80. Hanf (54), in 1927, observed the greatest frequency between the ages of 50 and 54 years. Simons (144), in 1937, compiled 2,796 cases of cancer of the lung and found that 80 per cent occurred between the ages of 40 and 70 years. In 1926, Thomas (155) noted that "for centuries before and after the time of King Tut, down to the discovery of America, the average length of life was 18 years; at the time of the French Revolution it had increased to 33 years; at the time of our own Civil War it had advanced approximately to 45 years; at present it is about 57 years, and the longed for 70 may be reached within the next 50 years, maintaining the present ratio of medical progress. Ten of these added years have been contributed to in the last two decades." The importance of such facts, according to Fried (41), is evident in connection with the well known cancer age. Fried also said that the increase in cancer of the lung may be due to progress in hygiene and preventive medicine resulting in increased human longevity.

A higher frequency of cancer of the lung in the male than in the female was first recognized during the latter half of the nineteenth century (115). This variation has persisted until the present. However, Kaufmann (74) reported that in Basle cancer of the lung was more frequent in the female than in the male. This difference in frequency between the male and female has served as one of the major factors in the attempt to establish an etiology for this particular neoplasm and to account for its progressive increase. Pässler (115), in 1896, in a group of 68 pulmonary neoplasms, found 50 males and 18 females. In the 374 cases collected by Adler (2) in 1912 the percentage of males was 71.9. In Weller's (164) group in 1913 there were 70 males and 17 females. Biberfeld (15) collected 600 cases from the literature and found the ratio to be 3 to 1. Breckwoldt (21), in 1926, in a group of 1,087 cases found the ratio to be 2.88 to 1. In 1931, Verga and Botteri

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(158) compiled 9,845 cases of lung cancer and found an incidence of 76.2 per cent for the males. Simons (144), in 1937, tabulated 5,121 histologically proved cases and found a ratio of 4 to 1 in favor of the male. Thus, we find the ratio of pulmonary cancer in the male and female to be 3 or 4 to 1 during the period from 1900 to 1930.

The increase in cancer of the lung that began in the nineteenth century had reached such proportions by 1910 to 1915 that every effort was being put forth to explain it on an etiologic basis. An association between pulmonary cancer and tuberculosis had been observed frequently before 1900 as well as after this date. The theory of an incompatibility between tuberculosis and pulmonary cancer, as advanced by Rokitsansky (130) in 1855, apparently was not correct. Friedländer (42), as early as 1885, had observed lung cancer in the wall of tuberculous cavities. Similar observations were made by Schwalbe (138) and Perrone (118). Barron (8), in 1922, in discussing the etiology of lung cancer said, "Perhaps the chief etiologic factors are inflammatory conditions, and of these, tuberculosis is the most important." Cherry (27), in 1925, expressed the opinion that "cancer is in most cases the expression of the resistance of the cells to a second or subsequent invasion by the bacillus tuberculosis." Ewing (34), as late as 1928, considered tuberculosis as the cause of pulmonary cancer. Syphilis, like tuberculosis, was also considered to be the etiology of lung cancer; however, the association was less impressive than that of tuberculosis (98, 122).

The early observations on tuberculosis and syphilis prepared the way for the theory of chronic irritation as the etiology of lung cancer, a theory that gained considerable support after 1910 and continued to be the prevailing one long after 1930. Adler (2), in 1912, said, "Chronic irritations affecting the respiratory organs are numerous and are supposed by many to play a very active part in the causation of tumors of the lung." If irritation is accepted as an important etiologic factor, carcinoma of the lung would be in line with many other forms of cancer (145).

Closely associated with the hypothesis of chronic irritation was that of metaplasia. Wahl (160), in 1927, emphasized this relation when he said, "We see therefore that in epithelial metaplasias the chronic irritations play a deciding part." Pässler (115), in 1896, pointed out the possibility of a relationship between meta-

plasia and cancer of the lung. Watsuji (162), in 1904, observed that 32.3 per cent of all pulmonary carcinomas that he investigated was "built up" from pavement epithelial cells. Subsequent investigators have pointed out the frequency of squamous cell carcinomas originating from the columnar epithelium lining the pulmonary bronchi. As late as 1927, Katz (73) had this to say referable to metaplasia and cancer, "The link in which the fact of chronic inflammation is connected with the observation of the subsequent cancer, which at first was of a purely temporal nature but is now recognized as causal, is closed: the metaplasia . . . . There is no doubt that the process of metaplasia creates a ground for the cancerization. And it is likewise undeniable that this metaplastic process is the result of a chronic state of irritation. Yes, one can say: that metaplasia is a precancerous state, without saying that now a carcinoma must always develop from each metaplasia."

Influenza received considerable attention after 1918 as a specific agent in the etiology of lung cancer. Askanazy (4), in 1919, apparently was the first to point out that metaplasia may occur in the lungs in influenza. He warned, however, against overestimating its role in the etiology of lung cancer for "individual cases of lung cancer one cannot know whether the metaplasia is a cause or a consequence of tumor formations." Askanazy's observation of metaplasia in the lungs after influenza was confirmed by Schmidt-mann (136) and Teutschlaender (154). Bauer (9), in 1921, was one of the first to report a case of pulmonary carcinoma, giving "grip" as a significant factor in the history. Meyer (105), in 1922, also reported a case of lung cancer following "grip." Moise (107), in 1921, Barron (8), in 1922, and Berblinger (12), in 1925, thought influenza was a primary factor in the increase of lung cancer. Winternitz *et al.* (170), in 1920, Wahl (160), in 1927, Katz (73), in 1927, and Huguenin (62), in 1928, concurred in this opinion. Simpson (145), in 1928, in discussing this problem of influenza and lung cancer had this to say, "The fact that the increase continued for several years after the influenza epidemic cannot be considered to rule out its aetiological importance, as it is known that irritative factors may not produce results until some time after they have ceased to operate."

Apparently there were as many investigators who rejected the theory of the relation of influ-

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enza to cancer of the lung as accepted it. Gottstein (48), in 1923, found no relation between "grip" and lung cancer. This opinion was concurred in by Staehelin (148) in 1925 and Hueper (61) in 1926. Seyfarth (141), Kikuth (76), and Hoffman (58) found only a few histories of influenza in their cases of lung cancer. Krompecher (84) maintained that no increase in lung cancer resulted from influenza in the Budapest material collected up to 1924. Probst (124), Wahl (160), and Mönckeberg (109) discussed this problem of the relation of influenza to lung cancer. The former pointed out that since lung cancer had been increasing almost continuously since 1910 in Zurich he could not support the idea that the increase was due to influenza. Hueper (61) did not find an increase in the incidence of pulmonary cancer after the epidemic of 1889-1894. Niskanen (111) wrote in 1949 that cancer of the lung was rare in Iceland although the epidemic was most severe. As late as 1948, Fried (40) said, "The conception that the alleged increase in bronchiogenic cancer is a sequel to influenza cannot be relied upon."

Interest in the etiology of lung cancer between 1900 and 1930 continued to center around infectious processes that produced chronic irritation with an associated metaplasia. McKenzie (101) and Haythorn (55) had observed metaplasia after pneumonia in 1907 and 1912. Siegmund (143), in 1922, reported 3 cases of pulmonary cancer in which there was bronchiectasis which he thought resulted from measles, whooping cough, grip, pneumonia, and chronic bronchitis.

The study of the pulmonary lesions in the Schneeberg mines during the latter part of the nineteenth century emphasized the importance of pulmonary irritation produced by non-bacterial agents as a possible etiology of pulmonary cancer. Pulmonary irritants associated with specific and nonspecific types of dust, chemicals, and irradiation, as observed in the Schneeberg mines, also were studied between 1900 and 1930 in an attempt to establish their relation to the increase in pulmonary cancer. Rostoski, Saupe, and Schmorl (133), in 1926, stated that 70 per cent of the miners that developed pneumoconiosis subsequently developed lung cancer. Staehelin (148) pointed out that miners elsewhere in the world showed no increase in the incidence of pneumoconiosis and pulmonary cancer. Rostoski (132), in 1924, noted that

pneumoconiosis leads more frequently to tuberculosis than to lung cancer. Uhlig (157), in 1921, thought that the inhalation of cobalt was really the cause of the lung cancer in the Schneeberg miners, but Heilmann (56), in 1925, thought it was due to the inhalation of stone dust. Berblinger (12) questioned whether nickel, cobalt, or arsenic was the real cause of these pulmonary cancers. Analyses of the lungs of the miners revealed calcium, magnesium, alum, silicic acid, chlorides, phosphates, but not a trace of arsenic, cobalt, bismuth, nickel, or uranium (13, 157). Risel (129) suggested that a gaseous arsenic combination resulting from fungi and oidia might be the cause of these pulmonary neoplasms. Schulte (137), after reviewing 487 cases of pneumoconiosis without finding one pulmonary carcinoma, suggested that in the Schneeberg mines the pulmonary cancers probably resulted from arsenicals coupled with the inhalation of large amounts of radium emanations. Others considered only radium emanations to be the cause of these neoplasms (123). Ludewig and Lorensen (93) found radium emanations up to 50 Mache units per liter of air in one of the Schneeberg mines and Santholzer (134) found up to 52 Mache units per liter of air in one of the Jachymov mines. Katz (73), in 1927 and after reviewing this problem, concluded that the etiology of the pulmonary lesions in the Schneeberg mines is an "unsolved problem."

Many other pulmonary irritants were carefully evaluated between 1920 and 1930 as possible etiologic agents for lung cancer. Hampeln (53), in 1923, proposed that lung cancer is a dust disease. Materna (99) in 1924, agreed with this opinion. Heilmann (56) showed that the pathologic changes in the lung produced by dust ultimately produced lung cancer. Berblinger (12), however, did not consider dust of itself a cause of lung cancer. Seyfarth (141) denied that any one kind of dust—street dust, metal dust, glass dust, or coal dust—could be held solely responsible for the increase in lung cancer. Probst (124) failed to find cancer of the lung more frequent in individuals such as road workers, drivers, policemen, chauffeurs, whose occupation exposed them to street dust. The observation that pulmonary cancer occurred in outdoor workers (33, 145) more frequently than in professional groups (23) emphasized an environmental factor to which males were exposed as the etiologic agent for cancer of the lung.

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According to Probst (124), Block in a lecture before the Association of Swiss Road Experts in 1924 discussed street tarring and suggested that it might be significant in the etiology of carcinomas in general. Stachelin (148), however, in 1925 could find no connection between the increase in the tarring of roads and the increase in carcinoma. Probst, in 1927, expressed a similar opinion. Vincent (159) thought that he could see a parallelism between the increase of cancer in general and the tarring of roads. Probst, in 1927, considered it questionable that street tarring alone had any causal relation to pulmonary carcinoma. Simpson (145), in 1928, expressed the opinion that "when one considers the widespread practice of tarring, the known potentialities of tar as an irritant factor, and the undue proportion of males in outdoor workers, it is obvious that here lies an urgent problem that demands scientific solution." Simpson regarded the rarity of lung cancer in Hong Kong and Singapore as due to the absence of tarred roads. Heilmann (56) thought that the increase in the frequency of lung cancer could be attributed to the effects of tar. Konrad and Franke (82) denied that the increase of the disease in Riga was due to either an increase in automobile traffic or the use of tar.

In 1925, the automobile was brought into this problem of lung cancer, not only from the fact that it ran across tarred roads but also because of its exhaust gases (56). Klotz (80), in 1927, called attention to the fact that "the cancer incidence of the lungs shows a parallelism with the increased use of gasoline engines, but we have no data that these two are in any way related." Probst (124) pointed out that coal tar fractions below 230 degrees under 1 to 2 mm. of Hg pressure contain little or no carcinogenic substance and that "the most active fractions were the ones between 250 and 293 degrees under 1 to 2 mm. of H pressure." The exhaust products of carburetor motors consist chiefly of carbon dioxide, nitrogen, water vapor, and varying amounts of carbon monoxide, hydrogen, and methane. Tars that are obtained from petroleum-like products exhibit an aromatic nature only when the decomposition temperatures are very high (900 degrees and above). It would appear that exhaust gases do not contain any compounds that are related to the tar constituents with which cancer has been produced in experimental animals (124).

Experimental observations were begun in 1923 and were continued for many years thereafter in an attempt to establish whether tars would produce cancer, especially pulmonary cancers. Kimura (78), in 1923, claimed to have produced cancer of the lung in a guinea pig following the intrabronchial insufflation of coal tar. Bonne (17), in 1927, injected coal tar intratracheally into 104 mice but did not observe any increase in lung cancer. Murphy and Sturm (110), in 1925, painted the skin of mice with coal tar and got an increase in pulmonary tumors. Möller (108) and Smith (146) exposed mice to coal tar fumes, automobile exhaust, and gasoline vapors, but found no increase in the frequency of lung cancer.

Specific chemicals have been suggested as the cause of lung cancer. Among these may be mentioned war gas (1). However, Hoffman (58), in 1929, said there was no evidence that exposure to irritant gases, including war gas, had been productive of lung cancer. Kikuth (76) reported a case of a pulmonary cancer in a chemist and suggested that chlorinated hydrocarbons could be held responsible. Certainly these isolated cases of lung cancer attributed to specific chemicals would not be a significant factor in the over-all increase of pulmonary cancer. Since this fact was realized many years ago, other etiologic agents were diligently sought for; among these were smoke and chemical impurities in the atmosphere (53, 56). Duguid (33), in 1927, felt that it "must be some specific constituent of the pollution rather than the general atmospheric pollution itself that should be suspected, because the atmospheric conditions in cities are generally supposed to be improved since the coming into operation of the Smoke Abatement Acts (England), and were it simply a matter of a smoke-laden atmosphere, the incidence of thoracic tumors would be expected correspondingly to be on the decrease."

As early as the end of the nineteenth century Soemmering (147) suspected smoking to be associated with pulmonary cancer. At the turn of the century Brosch (24), apparently the first, succeeded in producing malignant proliferation in the skin of guinea pigs by smearing the skin with "tobacco juice." Geipel (44), in 1899, Seyfarth (141), in 1924, and Kikuth (76), in 1925, noted cases of lung cancer in cigar makers. In 1912, Adler (2) had this to say about tobacco as a factor in the etiology of lung cancer, "The do-

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mestic life led by women, with their consequent retirement and immunity from the irritations and traumatisms which must be frequent in the more unprotected life of men (the abuse of tobacco and alcohol, the many trades and vocations which are accompanied by irritations of the respiratory organs, etc.) has been adduced in explanation of this fact." Fahr (35), in 1923, and Seyfarth (141), in 1924, associated cigarette smoking with cancer of the lung. Fahr thought the inhalation of cigarette smoke, which was becoming more frequent, would explain the increased frequency of lung cancer in the male. Staehelin (148), in 1925, expressed the opinion that "the mere fact that there are more men than women smokers and more male than female cases of lung cancer is not sufficient proof of the etiologic influence of smoking." Kikuth (76), in 1925, found a history of smoking infrequently in his cases and expressed the opinion that "pulmonary carcinoma would have to occur much more frequently among those who smoke to excess before this could be regarded as a big factor." Katz (73), in 1927, noted that cancer of the lung was not particularly frequent in the Orient where there was heavy smoking. Huguenin (62), in 1928, in discussing the relation of smoking to cancer said, "Beside the fact that this argument could cause a smile today, no fact of investigation any longer supports the role of smoking or tobacco intoxication." Hoffman (58), in 1929, stated, "There is no definite evidence that smoking habits are a direct contributory cause toward malignant growths in the lung." McNally (103), in 1932, pointed out that the increase in lung cancer parallels the increase in the consumption of cigarettes and, because of this, one is certainly led to believe that cigarette smoking is an important factor in the increase in frequency of the disease. However, in 1934, Sweany said, "If there is any merit in this theory, it should be manifest in the female sex, where the increase in the use of cigarettes has been greater than in men. If, for example, the ratio of men to women 20 years ago was 3 to 1, and now in 1932 it is 1.5 to 1, the case is well nigh proved. So far, no such increase in ratios has appeared, for the sex ratio was and still is around 3 males to 1 female, similar to other malignant conditions other than those of sex differences. On the other hand, there are many patients with lung cancer who do not give a history of cigarette smoking at all" (153).

Few, if any, investigators today consider

trauma a factor in the etiology of lung cancer; however, as late as 1930 Wells and Cannon (167) reported a case of pulmonary cancer in which trauma was considered to be the etiology. In 1913, Weller (164) collected 89 cases of lung cancer from the literature, in 3 of which the disease was attributed to trauma; he said, "We may, therefore, be certain that gross trauma is not an important etiologic factor." As for all of the proposed etiologic agents for cancer of the lung, there were some who supported trauma, at least they reported cases in which trauma had occurred (6, 38, 76, 119, 141, 148), and others who objected to the theory of trauma and lung cancer (8, 34).

Heredity as a factor in the etiology of pulmonary cancer had its supporters and nonsupporters in the interval between 1900 and 1930. Adler (2), in 1912, expressed the opinion that the incidence of malignant growths of the lung did not appear to be seriously affected by heredity. Huguenin (62), in 1927, said, "Whatever opinion one professes with regard to the primary cause of cancer, its pulmonary localization does not seem to be the result of chance; hereditary changes or acquired ones govern the development of malignant tumors of the lung; such at least are the reflections which the discoveries of our predecessors suggest." Weller (163), in 1929, voiced the conviction that heredity might be a significant etiologic factor in the production of these neoplasms. He suggested "the development of carcinoma of the lungs may be found to be due to an inheritable intrinsic predisposition which may be activated by a variety of chronic irritative factors." Schwyter (139) found congenital malformations of the lung in the neighborhood of the growth in 6 cases and as a result concluded that heredity or developmental errors might be the cause of pulmonary cancer.

Although primary attention had been given to the etiology and frequency of lung cancer during the period 1900 to 1930, there also was an increasing number of papers discussing diagnosis and treatment. There was a diagnostic procedure being investigated before 1900 (128) that ultimately proved to be a most valuable one in lung cancer. This was the demonstration of microscopic particles in the sputum of individuals with pulmonary cancer. The early investigators usually used fresh unstained sputum; however, in 1910, Ballet (7) stained the smears with picro-

carmine. In 1913, Bezancon and de Jong (14) fixed the smears in a 1 per cent solution of chromic acid and then stained them with a polychrome stain. Sternberg (151), in 1923, and Homann (60), in 1929, suggested the technique of embedding the cells in paraffin and cutting sections. It was pointed out, however, that serial sections were necessary with the latter technique. Some investigators contended that sputum examination was not practical because of cell autolysis (83). Typical neoplastic cells were found in the sputum of 13 of 25 cases by Hampeln in 1918 (52). Brunn (26), in 1926, expressed the opinion that sputum should be examined thoroughly as several cases of carcinoma of the lung had been diagnosed by the finding of tumor shreds and cells.

The roentgen rays, first described in 1895, soon were used in the diagnosis of lung cancer. Leo (88), in 1898, diagnosed a metastatic osteosarcoma in the lung with roentgen rays. Immelmann (66), in 1899, pointed out that "the x-ray diagnosis of diseases of the lungs and of the pleura, among which principally pneumonia, gangrene, tumors, tuberculosis and pleuritis are capable of throwing shadows on the screen, is a diagnosis 'per exclusionem' in the true sense of the word." Walsham and Beale (161), in 1900, in discussing skiagraphy in the diagnosis of chest diseases did not mention roentgen rays for the diagnosis of lung cancer. After discussing the roentgenographic findings, in 1906, in lung abscesses, gangrene, pneumonia, emphysema, collapse of the lung, effusion, pneumothorax, and hydrothorax, Pfahler (120), Director of the Roentgen Ray Laboratory at the Medico-Chirurgical Hospital in Philadelphia, said, "The greatest field of usefulness of the roentgen rays in lung diseases is the study of tuberculosis." Otten (113), in 1906, was the first to report the use of roentgen rays in the diagnosis of primary lung cancer and his article is accompanied by illustrations of the roentgenograms.

Adler (2), in 1912, had this to say about roentgen rays, "It was not very long ago that Frankel wrote that the x-rays were of little service in the diagnosis of lung tumors. Since then the x-rays have become a most remarkable and efficient aid to diagnosis in general, and there exists the well-founded hope of their increasing efficiency as further improvements in the apparatus and advances in technique are made." McMahon and Carman (102), in 1918, ex-

pressed the opinion that "in most instances the roentgen findings in primary carcinoma of the lungs are pathognomonic of the disease, and may be the first to suggest the exact nature of the pulmonary lesions." Four years later Barron (8) stated that many authors found the roentgenograms either of negative value or at times misleading. Maclachlan (95), in 1923, likewise questioned the value of x-rays in the diagnosis of pulmonary neoplasms. Huguenin (62), in 1927, pointed out that one might say that repeated radioscopic examination could be a factor in tumor proliferation in the lung. Although the discovery and increased use of the roentgen rays coincided with the increase in lung cancer, there is no proof that x-rays are a cause of cancer, but the possibility should be recognized (144).

Although therapeutic use of the roentgen rays had been suggested, Barron (8), in 1922, expressed the opinion that thus far it was of little or no value in treatment. Davidson (30), in 1930, thought that the x-rays possessed a very definite value in treatment, but should be classed among the palliative remedies as the benefits therefrom were only temporary. Kerley (75), in 1928, could find "no reports dealing with radium therapy, although this would appear to be the only rational method of treating the disease when it is seen late or when its origin is in an upper lobe bronchus close to vital structures. With our present knowledge of radium, it is probable that heavy external radiation of the chest, properly administered, would at least ameliorate the distressing dyspnea which is so constant a feature of lung cancer." Goltz (47), in 1930, said, "The treatment for this condition demands early diagnosis and since carcinoma of the lungs is usually carcinoma of the larger bronchi, local cautery and possibly radium by a skillful bronchoscopist offers the best hope." Among general remedies for lung cancer, selenium and copper were used according to Atkinson (5); however, he pointed out that the intravenous use of lead appeared to be a promising method of treatment.

The technique of direct bronchoscopy, first described in 1898 by Killian (77), soon was recognized as a valuable aid in the diagnosis of lung cancer. Renon and his associates (127), in 1910, recommended the use of the bronchoscope and the taking of biopsies from bronchial tumors. Adler (2), in 1912, had this to say about the bronchoscope, "It cannot be denied that the

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field of bronchoscopy may be greatly extended by improvements in apparatus and in technique. It is, however, the writer's opinion that its usefulness in the diagnostics of lung tumors, at this present writing at least, is limited." Chevalier Jackson (68), in 1917, removed an endothelioma from the right bronchus with the bronchoscope. Barron (8), in 1922, while speaking of bronchoscopy and pulmonary biopsy said, "Both are so difficult to perform that their use is not to be encouraged at the present time." MacLachlan (95) only a year later expressed the opinion that bronchoscopy had proved to be one of the most valuable diagnostic aids as yet offered. Others expressed a similar opinion (91) (67).

Sicard and Forestier (142), in 1922, introduced lipiodol as an aid in the diagnosis of pulmonary lesions. Its advantages and disadvantages were discussed in articles by Brown (25) and Archibald and Brown (3).

Benda (11), in 1904, commented on the fact that cancer of the lung occupied a unique position, inasmuch as it was the only cancer that was absolutely beyond the reach of the surgeon. He went a step further and added that, no matter what progress surgery might make, it could never hope to deal satisfactorily with lung cancer, as it would always remain impossible to make the diagnosis early enough for any reasonable expectation of a cure by surgical interference. About 1912 Jacobaeus (69) was one of the first to employ thoracoscopy in the diagnosis of lung cancer. However, in 1930, Davidson (30) said that surgery had reached a stage in which the exploration of the chest presented difficulties no greater than those of opening the peritoneal cavity, but that thoracoscopy still had its advantages. The first pneumonectomy for cancer of the lung apparently was performed in 1910 by Kümmell (85); the patient died on the sixth postoperative day from a septic infection of the operative site. Lenhartz (87), in 1910, reported the removal of a lobe for pulmonary cancer. The first successful total pneumonectomy for pulmonary cancer was performed in 1933 by Graham and Singer (49).

In attempting to summarize the progress in our knowledge of cancer of the lung during the period from 1900 to 1930, it would seem that one of the most important contributions was the demonstration that this neoplasm was more frequent than it was formerly thought to be. Opinions, however, were divided as to whether

this increase was real or only apparent. Many agents were investigated as possible causes for the increase in frequency of lung cancer; some were proved to be unlikely, while others, such as cigarette smoking and air pollution, were carefully studied after 1930.

Significant advancements were made between 1900 and 1930 in the diagnosis and the treatment of pulmonary cancer. The use of x-rays was more or less perfected as a diagnostic procedure. Surgery was developed to such a point that the first successful pneumonectomy for lung cancer was performed in 1933. The clinical diagnosis of this lesion greatly improved after 1900. By 1930 the correct diagnosis of lung cancer was being made in approximately 50 per cent of the cases by the better physicians who had access to the latest diagnostic facilities, such as x-rays and cytological examination of sputum.

Scientific interest in cancer of the lung continued to progress after 1930 with emphasis on its frequency, etiology, and treatment. Few diseases have had a more exciting history than that of lung cancer.

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C O P Y

THE UNIVERSITY OF TEXAS - MEDICAL BRANCH

Galveston

October 8, 1958

Robert C. Hockett, Associate Scientific Director  
Tobacco Industry Research Committee  
150 East Forty Second Street  
New York 17, New York

Dear Mr. Hockett:

Under separate cover we are sending you 25 reprints on our recent article "Cancer of the Lung from 1900 to 1930." We won't worry about the fact that you didn't help with this job since I am going to discuss with you the possibility of assistance on a similar project.

You may recall that I discussed with you sometime ago a project, that we have approximately 50 per cent completed, that involves the recording of all available references on cancer of the lung up to the present time. In our files we have approximately 12 to 15 thousand reference cards. We would like to get this material together and make it available to those who are interested in this problem. What I would like to do would be to list these references in alphabetical order and check out significant articles that deal with specific topics and designate them in specific groups.

This will require considerable checking in the library and it may be necessary to check in the National Library in Washington and probably the library in New York. I understand the latter has a nice collection of books on cancer of the lung. After this material is compiled, I would be very happy to turn it over to the Tobacco Industry Research Committee for their distribution as complimentary material to those in this country and elsewhere who may find this material of value. I think that the medical libraries in the country might be interested in receiving a copy.

If the Tobacco Industry Research Committee would not consider it wise to send this out as a complimentary publication, I think we might look into the problem of recording it in Washington. I understand there is a place in Washington where such data may be filed and one who *desires* it may obtain same for a nominal fee.

At the present time I do not have any information as to the cost of publishing the data. We would complete the references so that they could be copied, thus reducing the cost of publication. I estimate that we could do this for \$7500.00, that is, complete the references and get them in final form for subsequent printing.

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Mr. Robert C. Hockett

October 8, 1958

Do you think your group would be interested in considering this project? If there is any additional data you would like to have, I will be glad to supply same.

In compiling these references, there is one additional historical study that we wish to complete - Cancer of the Lung from 1930 to 1956. This specific study would then be considered as being supported by your organization.

With kind regards,

Yours truly,

/s/ R.H. Rigdon, M.D.  
Professor of Pathology

RHR:k

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# THE RESPIRATORY SYSTEM IN THE NORMAL WHITE PEKIN DUCK

BY

R. H. RIGDON

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## The Respiratory System in the Normal White Pekin Duck

R. H. RIGDON

*Department of Pathology, The University of Texas Medical Branch,  
Galveston, Texas*

(Received for publication June 26, 1958)

THE observations in this paper have resulted from a study of neoplasms induced with a carcinogenic agent in the respiratory tract of the white Pekin duck (Rigdon, 1957). The anatomical findings in the duck's respiratory tract have made it necessary to establish the normal for this bird. The respiratory system in the white Pekin duck is similar to that in other birds; however, there are some interesting variations when compared with similar structures in the chicken (McLeod and Wagers, 1939) and in the turkey (Cover, 1953; Rigdon *et al.*, 1958). Some of these differences no doubt reflect physiologic functions.

This investigation was supported by research grants C-1469 (C5) from the National Cancer Institute of the National Institutes of Health, Public Health Service and Tobacco Industry Research Committee.

The respiratory tract in the bird has been studied extensively by Sappey (1847), Chauveau (1890), Bradley (1915), Locy (1916), Kaupp (1918), Gilbert (1939), Zietzschmann (1943), and Hazelhoff (1951). Sappey in 1847 gave an excellent description of the respiratory apparatus and emphasized certain variations in different types of birds. A large part of Sappey's monograph is devoted to a review of the literature, beginning with the observations made by Aristotle about 400 B.C. and including those of John Hunter in 1774. Harvey in 1651 was the first to describe carefully the air sacs. Juillet in 1911 published a treatise on the anatomical, embryological, histological, and comparative study of the bird's lung.

Few anatomical observations have been made on the respiratory tract in the duck (Chauveau, 1890; and McLeod and

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Wagers, 1939); however, the mechanism of respiration in the duck has been studied by several investigators (Sappey, 1847; Huxley, 1913; Orr, 1913; Paton, 1913; and Dooley and Koppanyi, 1929). McLeod and Wagers (1939) have an excellent discussion of the respiratory system in the chicken. They describe the nasal cavity, the infraorbital sinus and the pharynx and also give a detailed discussion of the larynx. McLeod and Wagers (1939) make several casual references to some of the variations observed in the duck as compared with those in the chicken. They note that the infraorbital sinus is larger in its anterior part and its opening is on a lower level and somewhat larger than that of the chicken. These investigators point out that in the male duck "a remarkable spherical bony box complicates the structure of the syrinx" which is called the *bulla tympaniformis*. Another interesting feature in the paper of McLeod and Wagers (1939) is the description of the respiratory system referable to other anatomic structures.

#### DEMONSTRATION OF THE AIR SACS

Latex\* was used to inject the respiratory tract. The birds were sacrificed and a cannula was put into the proximal third of the trachea. The head of the duck was attached to a ring-stand. A funnel filled with latex was placed approximately 12 inches above the level of the head and it connected by a rubber tube to the cannula. When the latex ceased to enter, the trachea was ligated and the duck was removed from the ring-stand. The tail was elevated over the head permitting the latex to fill the air sacs in the cervical region. The air normally present in the abdominal air sacs, when the latex entered, escaped into the cervical and clavicular

air sacs during the time of the injection. The presence of this air interfered with the filling of these air sacs with the latex.

After the feathers were partially removed from the posterior surface of the body, the duck was put into a container with a 4 percent solution of formaldehyde and a 4 percent solution of acetic acid. After a few hours in this fixative more of the skin and feathers were removed to permit better preservation. After 24 hours fixation the remaining feathers and portions of the wings and legs were removed. After 5 to 7 days the muscles were carefully dissected to demonstrate the latex in the air sacs.

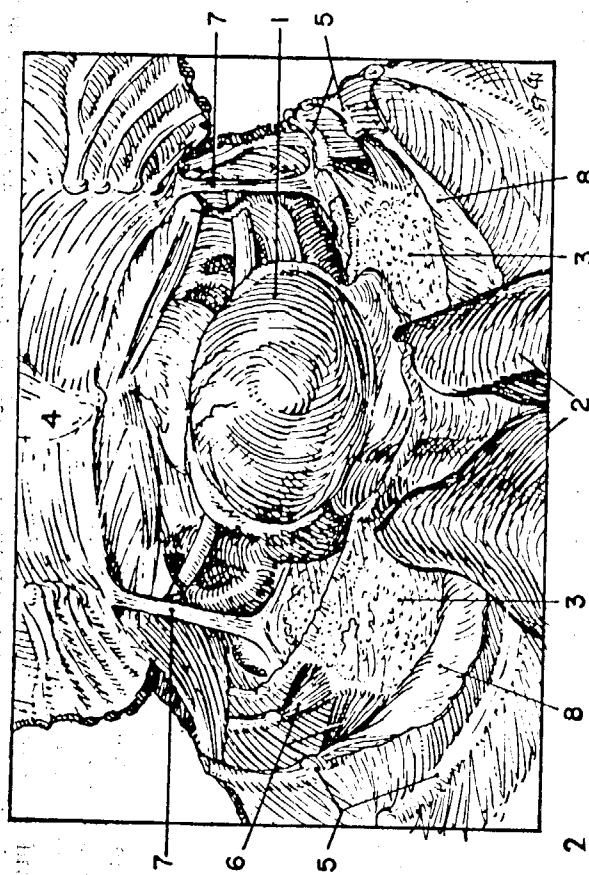
A circumscribed area in the cervico-thoracic region protrudes when pressure is applied to the abdomen (Figure 1). This area recedes when the pressure is removed. When the sternum and adjacent ribs are removed large spaces are present in the thoracic and abdominal cavities. These are the air sacs.

The lungs are present on each side of the thoracic cavity. There is no muscular diaphragm separating the lungs from the abdominal viscera only a thin membrane. The lungs are bound to the thoracic wall by fibrous adhesions and small muscles (Figure 2-6). These structures can be easily broken by blunt dissection and the lungs removed. The muscles are small and fan-shaped, extending from the ribs to the pleura and attached to the ribs along a line at the margin of the lung, extending 6 to 8 mm. over the surface of the lungs (Figure 2-5). In the area of the apex of each lung there is a muscle attached to the pleura and the inferior surface of the sternum. It measures approximately 25 mm. in length and 2-4 mm. in width (Fig. 2-7).

The posterior inferior surface of each lung is located at the anterior border of the abdominal air sacs (Figure 3-6). The

\* Polson Rubber Co., Garrettsville Ohio.

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esser abdominal air sac (Figure 3-6) is second in size to the greater abdominal air sac. It is located near the middle part of the lateral abdominal wall. The abdominal wall is lateral to this air sac and the greater abdominal air sac is medial. There are no diverticula from this air sac. The greater abdominal air sac (Figure 3-5) is the largest air sac in the duck, extending posteriorly the entire length of the abdominal cavity. Diverticula extend from the posterior superior surface of this air sac into the spaces about the head of the femur. The thoracic air sac is located at the inferior margin of the lung (Figure 3-7). The clavicular and cervical air sacs fill the anterior portion of the thoracic cavity (Figures 4 and 5). Diverticula from the periphery of the clavicular air sac extend out between the adjacent muscles and the subcutaneous tissue (Figure 2-4). The thoracic air sac on the right is larger than the corresponding air sac on the left (Figures 6-7). The cervical air sac on the right also is larger than its counterpart on the left (Figure 5). The posterior diverticulum is more conspicuous on the right cervical air sac than it is on the left. There is a single communication between the lung, the greater and the lesser abdominal air sacs on each side of the body. In the thoracic air sac there are 2 communications with the lung. In the clavicular

lar air sac there are 2 or 3 communications with the lung. Only one communication is present between the cervical air sac and the lung (Figure 7). There are no communications with each other between the different air sacs on a single side of the duck; however, the cervical air sac communicates with its counterpart on the opposite side through the cervical vertebrae and the clavicular air sac is continuous with its counterpart on the opposite side across the midline.

There is a communication between the clavicular air sac and the humerus through the *Foramen pneumaticum* which is located on the medial side of the proximal end of this bone (Figure 8). This foramen measures 1.5 cm. in diameter in the adult duck. There are many communications between the clavicular air sac and the sternum. Sometimes latex is present in the ribs near their attachment to the sternum. The cervical air sac communicates directly with the cervical vertebrae. Apparently this latex extends down from the cervical and thoracic vertebrae to infiltrate the lumbo-sacral mass (Figure 9). The thoracic vertebrae are diffusely infiltrated with latex. There are small collections of latex between the vertebrae on the anterior lateral surfaces. These collections are continuous with the latex within the vertebrae (Figure 10-2). There

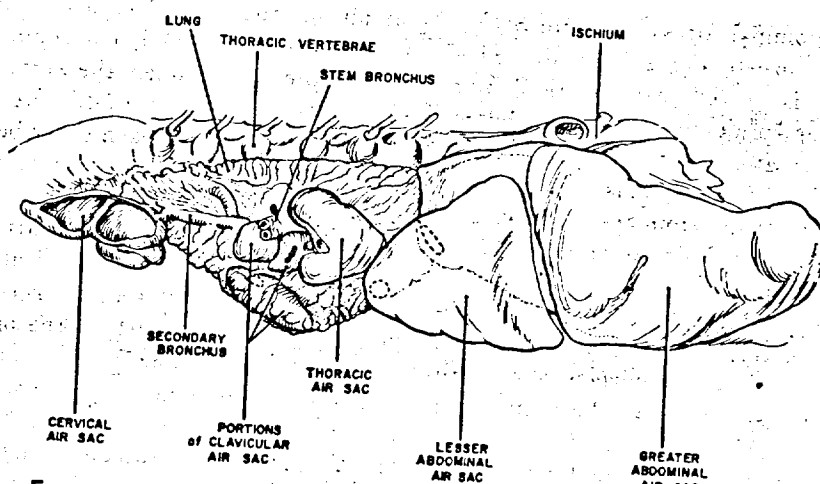
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FIG. 1. A circumscribed area is present in the cervico-thoracic area, after the feathers and skin are removed, that protrudes when pressure is applied to the abdomen. This area recedes when the pressure is removed.

FIG. 2. Drawing of thorax of the duck to show the small muscles (6 and 7) that extend from the pleural surface of the lungs to the bony thorax: (1) heart (2) liver, (3) lungs, (4) sternum-posterior surface, (5) ribs, (6) muscles between ribs and lung, (7) muscle between apex of lung and posterior surface of sternum, (8) wall of lesser abdominal air sac.

FIG. 3. Latex-filled respiratory tract of an adult white Pekin duck showing the left side: (1) trachea, (2) lung, (3) cervical air sac, (4) clavicular air sac, (5) greater abdominal air sac, (6) lesser abdominal air sac, (7) thoracic air sac.

FIG. 4. Latex-filled cervico-thoracic area in the duck with clavicular and pectoral muscles removed: (1) clavicular air sac, (2) axillary diverticula from the clavicular air sac, (3) coracoid bone, (4) sternum, (5) humerus, (6) cervical vertebrae, (7) trachea.

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are 2 latex-filled spaces of moderate size on each side of the midline in the area of the anterior portion of the lumbo-sacral vertebral mass (Figure 10-1). No latex has been observed within the lumen of the femur, the coracoid or the scapula.

OBSERVATIONS ON THE ANATOMIC  
AND HISTOLOGIC FEATURES OF  
THE RESPIRATORY TRACT

The trachea in the duck lies between the external larynx that opens into the oral cavity and its bifurcation which is located within the thoracic cavity (Figure 11). A circular cartilagenous enlargement is present in the male at the lower end of the trachea. This is the internal larynx or the syrinx (Figure 12-B). The external larynx is represented by a slit-like opening, the glottis located in the pharynx just posterior to the base of the tongue (Figure 13). The wall of the trachea is supported by cartilagenous rings which ossify during the first year of life. The trachea is lined by ciliated pseudostratified epithelium and has many mucin-secreting cells (Figure 14). Approximately one-fourth of the circumference of the trachea is lined by a cuboidal type of epithelium with no intervening mucin-secreting cells (Figure 15). Few lymphocytes are present in the fibrous tissue stroma between the lining epithelium and the cartilagenous rings of the trachea. Sometimes aggregates of lymphocyte form small nodules in this stroma (Figure 16).

The right and the left bronchus are approximately 2-3 cms. in length. After en-

tering the lung parenchyma the bronchi divide into primary, secondary, and tertiary or parabronchi. In some portion of the lung the larger bronchi are readily seen on the pleural surface (Figure 3-2). Some of the larger bronchi extend directly through the lung to communicate with the air sacs (Figure 7). The primary and secondary bronchi usually progressively decrease in size. The tertiary or parabronchi have a more or less uniform diameter throughout their entire length (Figure 17). Some of the parabronchi terminate as large spaces on the pleural surface of the lung (Figure 18), while others communicate with each other. A parabronchus and the adjacent air capillaries form the basic respiratory unit in the duck. These units usually form a 5 or 6 sided prism (Figure 19). In our latex-injected specimen the air capillaries did not fill.

The primary and secondary bronchi are lined by columnar epithelial cells similar to those in the trachea. A narrow band of smooth muscle is present about the larger bronchi and it continues into the wall of the parabronchi. Muscle is also present about the terminal dilatations of the parabronchi at the periphery of the lung. The parabronchi (Figure 20) and the small air capillaries are lined by endothelial-like cells (Figure 21-22). The spaces at the surface of the lung formed by the parabronchi (Figure 18) are also lined by either cuboidal or columnar epithelium. The stroma about the terminal

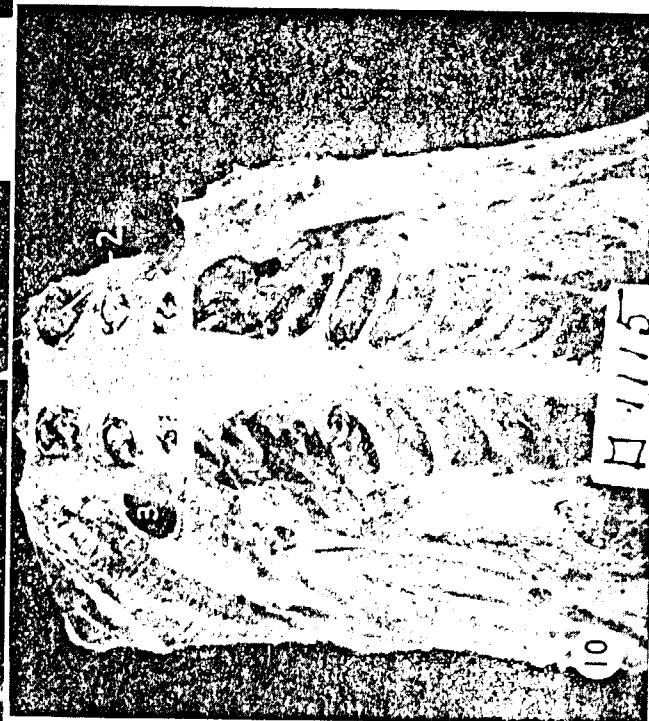


FIG. 5. Drawing to show the air sacs in their relation to the lungs.

FIG. 6. Latex-filled air sacs in the duck. Anterior view with sternum and viscera removed: (1) trachea, (2) syrinx in male duck, (3) bronchi, (4) clavicular air sac, (5) lesser abdominal air sac right, (6) portion of the right greater abdominal air sac (the greater part of this sac has been removed), (7) right thoracic air sac, (8) small air sac on left bronchus, (9) left lesser abdominal air sac, (10) coracoid bone, (11) axillary diverticulum from clavicular air sac, (12) cervical air sac, left.

FIG. 7. Latex-filled ventral bronchi showing a communication of one bronchus with cervical air sac (A)

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parabronchi is loose connective tissue. Only a few fibroblasts and a small amount of fat are present in this area of the lung. Small spaces lined with endothelial-like cells are suggestive of lymphatic channels.

To study the blood capillaries in the lung, an adult bird was sacrificed and saline was immediately injected into the pulmonary artery. When the saline returned through the pulmonary veins free of red cells, India ink was injected into the pulmonary artery. When the ink returned through the pulmonary veins, the entire carcass was fixed immediately in a 4 percent solution of formaldehyde. Sections of the lung were prepared by the paraffin technique for histologic study.

There are numerous blood capillaries about the small air spaces in the lung of the duck (Figure 23). These unite to form small vessels within the respiratory unit and then pass to the interlobular spaces uniting with the vessels from the adjacent lobules. Many cross sections of nerves are present in the histologic sections of the lung. Small ganglia are also present. It is of interest to find in the osmic acid stains that all the nerve fibers are not myelinated. Pacinian corpuscle is sometimes demonstrated in the wall of the trachea (Figure 24). An occasional microscopic mass of osteoid tissue is present in the lungs.

#### DISCUSSION

There are 9 air sacs in the white Pekin duck; the clavicular is single while the cervical, thoracic, lesser and greater abdominal air sacs are in pairs. The number of air sacs in the duck corresponds with that given by McLeod and Wagers (1939) for the chicken. In the turkey, Cover (1953) and Rigdon *et al.* (1958) found only 7 air sacs. The terminology used for the different air sacs in birds varies (McLeod and Wagers, 1939).

In the duck, the humerus, sternum and vertebrae are pneumatized. The humerus and sternum communicate with diverticula from the clavicular air sac, while the cervical vertebrae are in direct communication with the cervical air sacs. Apparently air diffuses from the cervical vertebrae in the duck into the other parts of the vertebral column. Kaupp, in 1918, pointed out that the cervical air sac furnished air to all the cervical and dorsal vertebrae and to all the vertebral ribs. He also said the air "after passing through the first vertebra, leaves by a lateral exit to enter a small air sac. From this it passes into the superior part of the second vertebra escapes from this through its lower portion, to be received into a lateral sac and so on to the last dorsal vertebra." In the duck the air continues from the last dorsal vertebra into the sacrum (Figures 8 and 9). In the domestic fowl "the ab-



FIG. 8. The latex has been removed from the foramen pneumaticum in the proximal end of the humerus. (A portion of the cortex of the humerus has been removed to show the latex-filled lumen.) A diverticulum from the clavicular air sac communicates with the lumen of the humerus through this foramen.

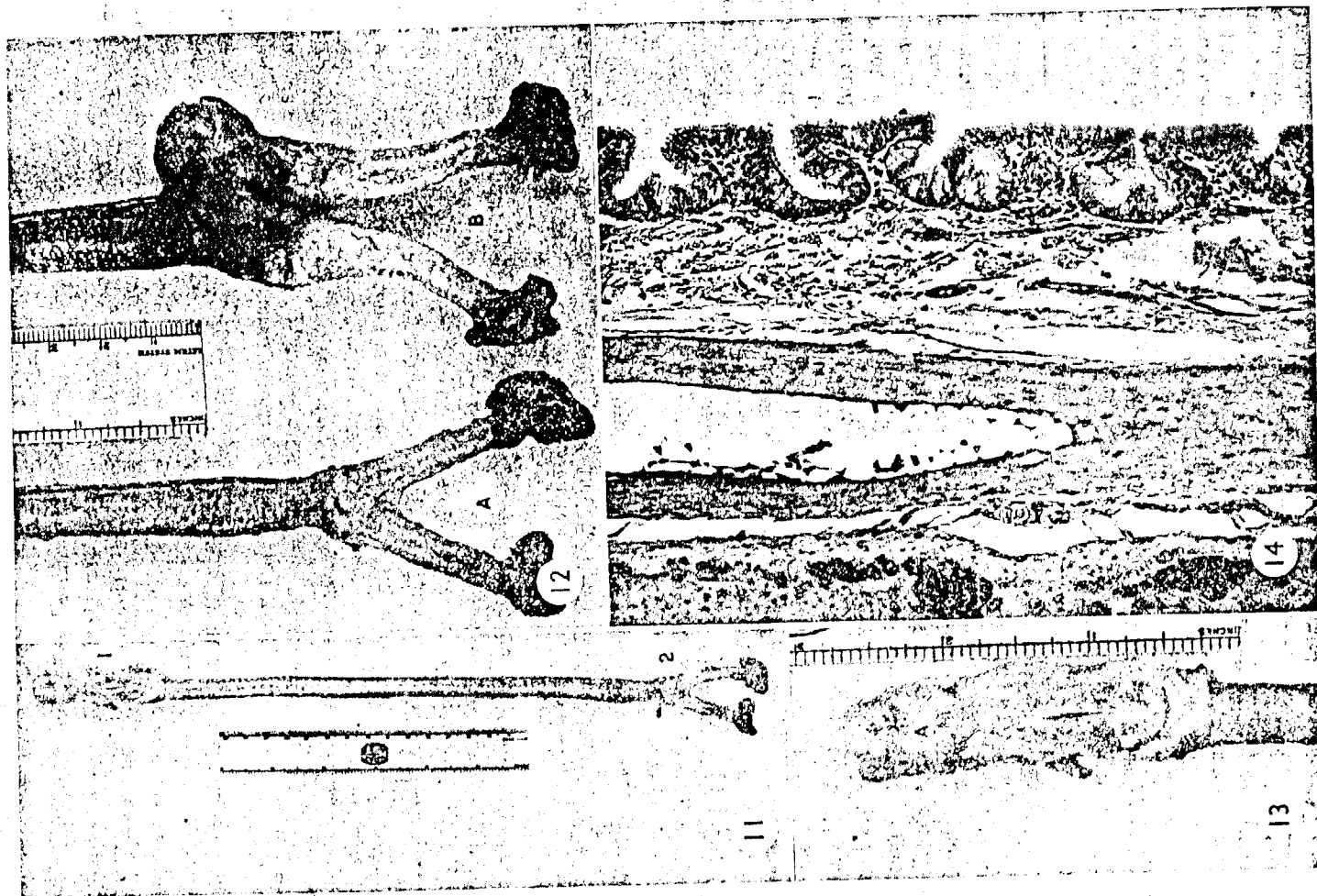
FIG. 9. Cross sections of the thoracic vertebra and the lumbo-sacral mass. All of these vertebrae are filled with latex. Apparently the air that enters the cervical vertebra diffuses downward to fill the remaining vertebra: (1) spinal cord, (2) latex in a thoracic vertebra (latex fills the spaces in the vertebra, however, it does not show too well in this photograph), (3) head of femur, (4) air sac on each side of the vertebrae.

FIG. 10. Anterior surface of the lumbo-sacral mass. An air pocket (1) is located on each side of the vertebra superior to the kidney. It extends upward to the junction of the last thoracic vertebrae with the ilium. Note the air pockets filled with latex (2) between each thoracic vertebra. (Diverticula from the greater abdominal air sac pass through the sciatic foramen (3) and end blindly around the head of the femur).

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R. H. RIGDON

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dominal air sacs communicate with the sacrum, the coccygeal vertebrae, the iliac bones and the femur" (Kaupp, 1918). The air passing to the above 3 bones, according to Kaupp (1918), comes directly from the suprarenal extension, while the air that fills the femoral cavity comes from the femoral extension which arises from the abdominal air sac. Diverticula from the suprarenal air spaces in the duck extend through the sciatic foramen and end blindly around the head of the femur. Other diverticula extend directly from the greater abdominal air sac in the duck to the tissues around the head of the femur. The femur itself has been observed to be pneumatic in some birds (Sappey, 1847). Foust (1952), however, did not find any evidence of pneumatic cavities in the femur of a large number of birds that he studied. Sappey (1847), pointed out that the suprarenal prolongations are not present in all birds.

The bones that are always aerated in all birds, according to Kaupp (1918), "are the cervical and the dorsal vertebrae, the sternum, and the humeri. Those aerated in some birds only are the furculum, the scapulae, the vertebrae and the sternal ribs, the sacrum, the coccyx, and the femurs. Those that are never aerated are the bones of the forearm, the hand, the leg, and the foot. The Eustachian tubes furnish air to the bones of the cranium and to the upper jaw; while the lower jaw receives air from the pneumatic foramen situated upon each ramus behind the tym-

panic articulation, and from an air cell which surrounds the joint. Selenka states that the invasion of the bones by the air is a late development, and that in the humerus this invasion occurs after the twenty-second day in the life of the chick."

In the chicken, McLeod and Wagers (1939) have described 3 groups of recurrent bronchi, 2 of which arise from the abdominal air sacs and the other from the lateral part of the so-called anterior thoracic air sac. In the duck, only one major communication was demonstrated between each of the greater and lesser abdominal air sacs and the lungs. It is also of interest to find that in the duck the parabronchi frequently terminate as large spaces on the surface of the lung. In the chicken, McLeod and Wagers (1939) say, "May we repeat that there are no blind ends in the bronchial system of the bird. The air passages are continuous and have numerous anastomoses."

Considerable confusion exists in the literature concerning the presence or the absence of a diaphragm in birds. McLeod and Wagers (1939) consider that 2 are present: the thoraco-abdominal and the pulmonary. There are thin fibrous membranes which may be considered as diaphragms in the duck. There is also a thin membrane covering the surface of the lungs in the duck, which may be compared to the pleura in mammals.

A number of views have been expressed as to the mechanism by which air circu-

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FIG. 11. Trachea from adult female White Pekin duck showing the (1) external and (2) internal larynx.

FIG. 12. Bifurcation of the trachea of an adult duck: (A) female, (B) male. The internal larynx or syrinx in the male duck is a large cartilaginous-like box called the "bulla tympaniformis."

FIG. 13. The external larynx in the duck is represented by a slit-like opening in the floor of the pharynx surrounded by the glottis: (A) base of tongue showing row of papillae.

FIG. 14. Ciliated pseudo-stratified epithelial cells line the greater part of the trachea. Hematoxylin eosin stain,  $\times 150$ .

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lates through the lungs and air sacs of birds (Sappey, 1847; and Huxley, 1913). It is of considerable interest to find that the lungs of the white Pekin duck are bound to the thoracic wall by several small muscles. No doubt these play a significant role in respiration.

Sturkie (1954) has reviewed the role of the air sacs in respiration. It has been interesting to find that large quantities of fluid (270 ml. saline within an interval of 2 hours) may be put into the trachea of adult ducks and the birds show little respiratory difficulty. Apparently this fluid is rapidly removed from the respiratory tract. The mechanism of the removal of fluid and particulate matter from the lung of the duck will be reported in a subsequent study. The direct communication of some of the larger bronchi with the air sacs and the long, more or less uniform, diameter of the parabronchi frequently terminating in large spaces on the surface of the lung may be a significant factor in the mechanism of elimination of particulate material from the respiratory tract of the duck.

Lymphatic glands are absent in the duck. There are lymphatic channels in the lungs of birds that drain into the larger lymphatics which in turn communicate with the thoracic duct and then empty into the vena cava. Particulate material, when put into the trachea, usually can be demonstrated in the dilated parabronchi at the periphery of the lung

and in the larger air sacs. The mechanism of phagocytosis in the respiratory tract of the duck is now being studied.

There is nothing of particular interest observed in the histologic study of the respiratory tract in the duck. It is important to know that the cilia on the epithelial cells in the respiratory tract degenerate rapidly following death. Care must be exercised in expressing an opinion on the significance of the absence of cilia from the wall of the trachea. Similarly, the absence of myelin on some of the nerve fibers in the duck might be interpreted as a pathologic process; however, the frequency in which this has been observed in the ducks used in this study would indicate that normally some of the nerve fibers are non-myelinated. Kaupp (1918) has pointed out that nerves in domestic fowls may be divided into medullated and non-medullated. The non-medullated nerves "are confined to the gray matter and to the beginnings and endings of sheath axones, all the latter being uncovered for a short distance after leaving the nerve cell body and also just before reaching their terminations."

#### SUMMARY

The respiratory tract in the white Pekin duck has been described. It is similar to that in the chicken. There are 9 air sacs in the duck; a single clavicular and 4 pairs, cervical, thoracic, and lesser and greater abdominal. The location of these



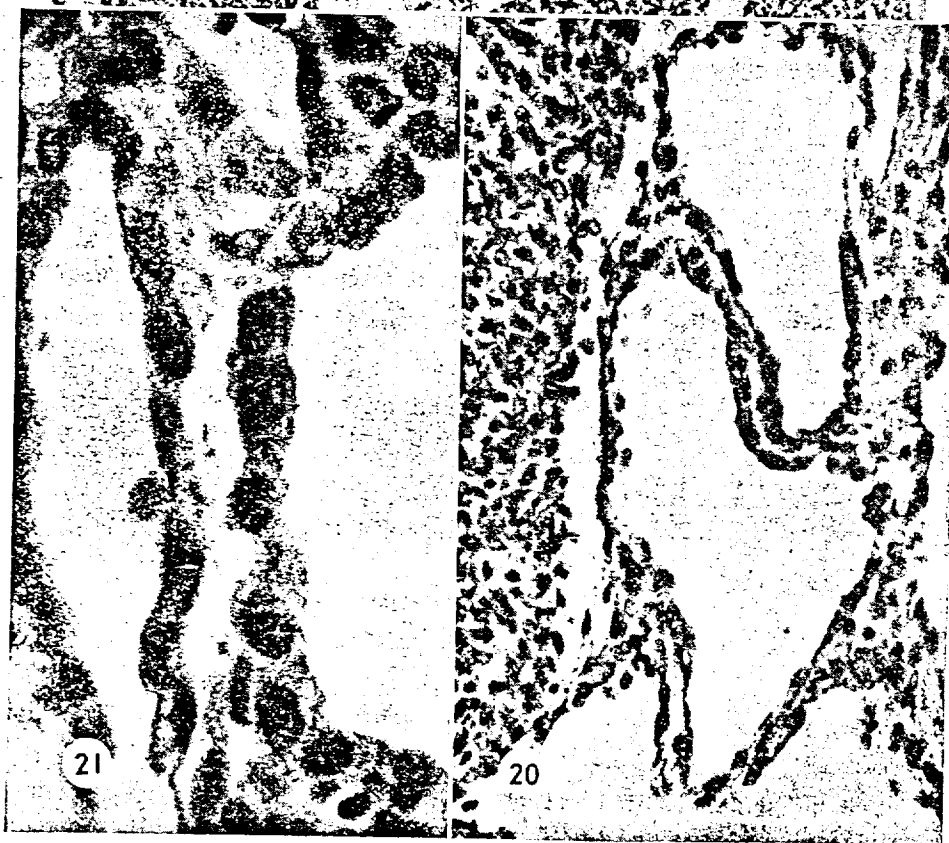
FIG. 15. A portion of the wall of the trachea is lined by a cuboidal pseudo-stratified layer of epithelial cells without mucin-secreting cells. Hematoxylin eosin stain,  $\times 150$ .

FIG. 16. Small groups of lymphocytes occur normally in the wall of the trachea. Hematoxylin eosin stain,  $\times 150$ .

FIG. 17. Tertiary or parabronchi in the lung of the duck filled with latex. The rough outer surface of these bronchi results from the failure of the latex to fill completely the surrounding air capillaries. Some of the parabronchi end in dilated spaces (1) on the surface.

FIG. 18. Latex-filled spaces on the surface of the lung represent the termination of some of the parabronchi.

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sacs is shown in the latex-injected specimens. The gross and histologic characteristics of the respiratory tract are described and illustrated. The capillaries in the lungs are shown in a preparation injected with India ink.

Small muscles attach the lung to the thoracic wall in the duck. These muscles may be significant in respiration in the duck.

The communication of some of the larger bronchi with air sacs and the termination of many of the parabronchi in large spaces at the periphery of the lungs create a reservoir for fluid and particulate material when put into the respiratory tract through the trachea.

Latex is a good medium to inject intratracheally to demonstrate the air sacs in the duck. Sometimes the bones are filled better in one bird than in another. This variation may account also for some minor diverticula being present in one duck and not in another. Such differences may represent only variations in ducks.

There are no lymph nodes in the duck; however, lymph channels are present in the lung.

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FIG. 19. A parabronchus with surrounding air capillaries: (A) parabronchus, (B) air capillaries. This is the basic respiratory unit in the lung of the duck. Hematoxylin eosin stain,  $\times 120$ .

FIG. 20. Thin septa are present within the lumen of the parabronchi. These septa are covered by endothelial-like cells. Hematoxylin eosin stain,  $\times 400$ .

FIG. 21. Large endothelial-like cells line the parabronchi. Hematoxylin eosin stain,  $\times 1140$ .

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FIG. 22. The lumen of the small air capillaries in the lung also is lined by endothelial-like cells. Hematoxylin eosin stain,  $\times 1140$ .

FIG. 23. Capillaries now filled with India ink surround the small air capillaries in the lung. Hematoxylin eosin stain,  $\times 1026$ .

FIG. 24. Pacinian corpuscle in the wall of the trachea of the duck. Hematoxylin eosin stain,  $\times 215$ .

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Memorandum to

Dr. Robert C. Hockett, Associate Scientific Director  
Tobacco Industry Research Committee  
5320 Empire State Building  
New York 1, New York


This is in response to your memorandum of January 5, requesting comments on Research Grant Application No. 120, Dr. Leo G. Rigler.

Unquestionably, the project would be well managed in the hands of such an outstanding roentgenologist as Dr. Rigler.

We raise the question as to the practicability of drawing a clear line of demarcation between "heavy smog areas" and "smog-free areas".

As laymen, we wonder if the small number of patients with carcinoma of the lung (10 to 20 in two years) is adequate to evaluate LDH and isomerase as an index to the disease or if, again considering the small number of patients, the variation in intensity of the hereditary factor might not outweigh the environmental factors included in this study.

Is there not also the question of the value of x-ray as a means of detecting cancer of the lung except in advance stages?

  
H. R. Hanmer  
For the Sub-Committee of the  
Industry Technical Committee

VH

cc: Dr. R. N. DuPuis ✓  
Mr. C. W. Baber

1003540942

RECEIVED  
JAN 12 1956  
PHILIP MORRIS & CO. LTD. INC.  
RESEARCH DEPT.

Memorandum to

Dr. Robert G. Hockett, Associate Scientific Director  
Tobacco Industry Research Committee  
2320 Empire State Building  
New York 1, New York

This is in response to your memorandum of January 2, requesting comments on Research Grant Application No. 120, Dr. Leo G. Rigler.

Undoubtedly, the project would be well managed in the hands of such an outstanding roentgenologist as Dr. Rigler.

We raise the question as to the practicability of drawing a clear line of demarcation between "heavy smog areas" and "smog-free areas".

In answer, we wonder if the small number of patients with carcinoma of the lung (10 to 20 in two years) is adequate to evaluate LDH and tobacco as an index to the disease or if, again considering the small number of patients, the variation in intensity of the heredity factor might not outweigh the environmental factors included in this study.

Is there not also the question of the value of x-ray as a means of detecting cancer of the lung except in advance stages?

H. R. Hanner  
For the Sub-Committee of the  
Industry Technical Committee

1003540943



TOBACCO INDUSTRY RESEARCH COMMITTEE  
350 FIFTH AVENUE NEW YORK 1, N. Y.

A. Budget Plan:

Salaries  
Application For Research Grant

#120

Overhead (11%)  
Other (Travel)

Date: December 6, 1955

1. Name of Investigator: 1. Leo G. Rigler, M.D.  
2. Norman Zheutlin, M.D.  
3. Borroughs Hill, Ph.D.  
7. Anticipated Duration of: 4. Howard R. Bierman, M.D.

2. Title: 1. Director, Division of Postgraduate Medical Education & of Radiology. (Prof. of Radiology, University of Minnesota, on leave of absence) 2. Radiologist  
3. Research Associate in Biochemistry of 4. Scientific Director; adequate  
13. Institution and out-patient facilities:  
& Address: City of Hope Medical Center  
Duarte, California

4. Project or Subject: A Study of the Development of Carcinoma of the Lung in Smokers and Non-Smokers Subjected to the Influence of Smog.

9. Additional Requirements: 1. Assistant Radiologist (half time) \$6,750; 2. Physician \$8,500;  
3. Radiology Technicians (2) \$8,000; 4. Radiology Technician \$4,500; 5. Experimental  
Supplies, Glassware and chemicals \$2,500, x-ray film \$2,500, gas and oil \$500;  
6. Equipment: fluoroscopic x-ray unit (1000) in a mobile van completely  
equipped for radiography and laboratory work \$25,000 (first year only).

5. Detailed Plan of Procedure (Use reverse side if additional space is needed): The increased development of carcinoma of the lung in man is related to many factors, one of which has been alleged to be heavy cigarette smoking (1,2). Patients with carcinoma of the lung have, in large measure, been found to be exceptionally heavy smokers as compared with control groups (1,2). If cigarette smoking is truly a major causative factor in the development of carcinoma of the lung, it is surprising that a much higher incidence has not been found, strongly suggesting that other factors may be intimately involved (3). The relatively high rates of carcinoma of the lung in urban areas as compared to rural areas demands a thorough scrutiny of air pollution by smog and possibility of the activity of toxic acid components and irritants. It may be possible to detect subclinical cases in their early pathological state. It would appear necessary, therefore, to determine the influence of a combination of environmental and host factors such as: cigarette smoking, air pollution (smog), heredity, occupation, etc. upon the development of pathoses in the lung (3). Summary of this project was reported in Cancer 62, May 1953.

It is proposed to study carefully 10,000 male subjects, 45 years of age or older, for a period of at least five years. A group will be selected with strong hereditary cancer histories and other similar attributes which would favor an increased incidence of carcinoma of the lung. The incidence of carcinoma of the lung in 4,000 heavy smokers (two packs or more a day) from the smog areas would be compared with a control group of 4,000 heavy smokers in smog-free areas, 10,000 non-smokers in heavy smog areas and 1,000 non-smokers living in the surrounding environs of Los Angeles out of the smog area. The subjects will be selected from the aircraft manufacturing and other industries where the population is stable, willing to cooperate, and available for study by a mobile x-ray and laboratory unit.

Air pollution in the form of smog has been shown to have carcinogenic properties (4a) and the possibility of a co-carcinogenic action between smoking and smog in man (4b) is a

a/ Nori Brandler

Business Officer of the Institution

Assistant to Executive Vice President and  
Financial Officer

1003540944

6. Budget Plan:

Salaries (Inc. 2% Soc. Sec.)	\$27,489
Expendable Supplies	9,800
Permanent Equipment	29,000
Overhead (15%)	9,943
Other (travel)	500
<b>Total</b>	<b>\$76,732</b>

Date December 6, 1955

1. Name of Investigator: 1. Leo G. Rigler, M.D.
2. Norman Zheutlin, M.D.
3. Harrold Hill, Ph.D.
7. Anticipated Duration of Work: 5 years minimum

2. Title: Director, Division of Postgraduate Medical Education & of Radiology. (Prof. of Radiology, University of Minnesota, on leave of absence) 2. Radiologist  
3. City of Hope Medical Center Division of Research. 27 full time people; adequate laboratories and out-patient facilities.  
4. Address: City of Hope Medical Center  
Duarte, California

4. Project or Subject: A Study of the Development of Carcinoma of the Lung in Smokers and Non-Smokers Exposed to the Influence of Drug.

9. Additional Requirements: 1. Assistant Radiologist (half time) \$6,250; 2. Physician \$8,500; 3. Biochemistry Technicians (2) \$8,000; 4. Radiological Technician \$4,200; 5. Expendable Supplies: Glassware and chemicals \$2,300, x-ray film \$7,000, gas and oil \$500; 6. Permanent equipment: Photofluorographic x-ray unit (4x5) in a mobile van completely equipped for radiography and laboratory work \$29,000 (first year only)

5. Detailed Plan of Procedure (Use reverse side if additional space is needed. The investigator may request an oral-10. Additional Information (including relation of work to other projects and other sources of supply):  
be heavy cigarette smoking (1,2). Patients with carcinoma of the lung. In 1952, an incidence of cancer in 460 per. out of a group of 188,079 persons interviewed in 1952, an incidence of cancer in 460 per. If 100,000 has been uncovered in heavy smokers. Thus it can reasonably be expected that there is a highly selected population of 4,000 heavy smokers (male) that at least 10 to 20 patients with carcinoma of the lung should become detectable by average clinical standards in the course of a two year period. By prescreening carefully in this group in addition to the predictability of the activity of lactic acid dehydrogenase and isomerase it may be possible to detect additional cases in their early subclinical state. It would appear necessary, therefore, to determine the influence of a combination of environment (This proposed research would be similar to the "Pulmonary Neoplasm Research Project", a preliminary report of which appeared in J.A.M.A. 157:440-444, Jan. 29, 1955. A summary of this project also appeared in Ca5:3-82, May 1955)  
It is proposed to study carefully 10,000 male subjects, 45 years of age or older, for a period of at least five years. A group will be selected with strong hereditary cancer histories and other similar attributes which would favor an increased incidence of carcinoma of the lung. The incidence of carcinoma of the lung in 4,000 heavy smokers (two packs or more a day) living in areas would be compared with a control group of 4,000 heavy smokers in non-high areas, 10,000 non-smokers in heavy smoke areas and 10,000 non-smokers living in the surrounding environs of low smoke areas out of the smoke areas. The subjects will be selected from the aircraft manufacturing and other industries w/Leo G. Rigler, M.D.'s stable, will try to cooperate, and available for study by the Director of Projection Laboratory Unit.

air pollution in the form of smog has been shown to have carcinogenic properties (4a) and the possibility of a co-carcinogenic action between smoking and smog in man (4b) is a

s/ Mort Brandler

Business Officer of the Institution

Assistant to Executive Vice President and  
Financial Officer

5. (continued)

vital question that needs a direct approach. The incidence of carcinoma of the lung in the Los Angeles County General Hospital has been carefully recorded by Steiner (5) and may be employed for comparative purposes. It would appear that the Los Angeles County area is ideal for such a study because of its large variegated population, the prevalence of smog, and the sympathetic interest of the inhabitants toward the problem. The City of Hope Medical Center is located 20 miles from the center of Los Angeles, equidistant from areas free of or heavily saturated with smog. The status of air pollution is available from the Air Pollution Control District.

Patients will be restudied at 4 to 6 month intervals. The plants will be visited by a mobile unit to take films and blood. Postero-anterior and lateral chest roentgenograms will be taken on these subjects on 4 x 5 cut film by photofluorography. Comparison studies will be made with a view to detecting minimal changes suggesting abnormali-~~ties~~ abnormalities. Re-examination with life size (14 x 17) films will be done on all cases showing abnormality. This would require approximately 60,000 minifilms and approximately 3,000 14 x 17 films per year. Intensive histories including hereditary, family, and social backgrounds will be obtained on all patients by a combined questionnaire and personal interview technic developed and employed at City of Hope Medical Center (6). Careful records of weight, smoking habits, etc. will be maintained.

The lactic acid dehydrogenase (LDH) activity of the serum of all patients will be determined on every other visit (7,8,9). The micro-technic permits the determination on finger tip blood. The lactic dehydrogenase activity has been found to be elevated in the sera of many patients with cancer and leukemia, but has not been raised consistently in other diseases. Significant ~~elevations have been found~~ elevations have been found in a number of very early cases of cancer and leukemia. This study of a selected group of ~~xxxxxx~~ individuals among whom the incidence of lung cancer may be expected to be increased over the average population over a five year period could afford valuable data in establishing the possible predictive value of this enzyme determination in cancer detection.

The ~~LDH~~ LDH technic was devised by Dr. Borroughs Hill, Research Associate in the Department of Biochemistry, and the laboratory and ~~equip~~ equipment for this determination are available.

The hexosephosphate isomerase level in the blood has been reported by Bodansky et al. to be elevated early in neoplastic diseases (10). Determination of the isomerase level will be done on all patients every other visit (each eight months). Some estimation of its value will also thus become available.

All patients in whom suspicious findings of pulmonary neoplasm were revealed would be studied more carefully for changes in pulmonary function employing the mass spectrometer technics developed by Tokuyasu, Coblentz, and Bierman (11), Papanicolaou's smears, bronchograms, planigrams, etc. in collaboration with the patient's physician.

Dr. Leo C. Rigler and Dr. Norman Zheutlin will supervise and assist in interpretation of films in an effort to detect the early development of carcinoma of the lung. Dr. Rigler has had extensive experience in film screening for large Public Health Service projects (12,13,14).

1003540946

# BIBLIOGRAPHY

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2. Doll, R., and Hill, A. B.: BRIT. M. J. 2:127, 1952
3. Berkson, J.: The Statistical Study of Association Between Smoking and Lung Cancer: PROC. STAFF MEET. MAYO CLINIC 30:319-347, 1955
- 4a. Kotin, P. and Falk, H.: Production of Tumors in C57BL Mice with Atmosphere-Extracted Aliphatic Hydrocarbons: PROC. AMER. ASSN. CANCER RESEARCH 2:30, 1955
- 4b. Stocks and Campbell: Investigation to Evaluate the Effects of Tobacco Smoking and Air Pollution by Benzpyrene Acting Together on the Men in Rural North Wales and Urban Liverpool: BRIT. M. J. 2:923, 1955
5. Steiner, P.: Cancer: Race and Geography: WILLIAMS AND WILKINS, 1954
6. Bierman, H.: A Comprehensive Self-taking History Method as an Aid to Physicians: In Preparation.
7. Hill, B. and Levi, C.: Elevation of a Serum Component in Neoplastic Disease: CANCER RESEARCH 14:513, 1954
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9. Bierman, H. and Hill, B., Emory, E., Reinhardt, L., and Samuels, A.: Correlation of Serum LDH with the Clinical Status of Patients with Neoplastic Diseases: PROC. AMER. ASSN. CANCER RESEARCH 2:5, 1955. In preparation.
10. Bodansky, O.: Serum Phosphohexose Isomerase in Cancer, I.: CANCER 7:1191, 1954
11. Tokuyasu, K., Coblenz, A., and Bierman, H.: Dynamic Partition of Alveolar Ventilation, Analyzed on Nitrogen and Helium Eliminations, by the Use of Mass Spectrometer: In preparation.
12. Newell, R., Chamberlain, W., and Rigler, L.: Descriptive Classification of Pulmonary Shadows: AM. REV. TUBERCULOSIS 69:566, 1954
13. Rigler, L., O'Loughlin, B., and Tucker R.: The Duration of Carcinoma of the Lung: DIS. OF THE CHEST 23:50, 1953
14. Rigler, L.: Natural History of Carcinoma of the Lung: To be published - ACTA RAD. INTERAMERICANA

1003540947

TOBACCO INDUSTRY RESEARCH COMMITTEE  
150 EAST FORTY SECOND STREET NEW YORK 17, N.Y.

#186

Application For Research Grant

Date:

December 6, 1957

Applicant:

1. Name of ~~Applicant~~: Walter F. Riker, Jr., M.D.
2. Title: Professor of Pharmacology  
Cornell University Medical College
3. Institution & Address: New York State Society for Medical Research  
2 East 63 Street  
New York, N.Y.
4. Project or Subject: I am writing to ask the support of the Tobacco Industry Research Committee for the New York State Society for Medical Research. I have outlined the essential points regarding our Society and its needs. I firmly believe that our needs are basic to medical research itself and merit consideration in the same way as any other application. I hope that this will serve as application for a grant-in-aid of \$5,000 for a one year period. This might be categorized as equivalent to a small grant application, but it is my conviction that its significance is more basic, broader and equally deserving. I trust that your advisory board will agree.
5. Detailed Plan of Procedure (Use reverse side if additional space is needed):
  1. The Society is a nonprofit organization of research scientists, physicians and laymen whose purpose is to encourage and facilitate research in the biological sciences.
  2. The Society was organized in the State of New York in 1950 to combat the threat of anti-vivisectionist legislation, which if successful, would have prevented medical research in all the renowned university, institutional and industrial laboratories within the state.
  3. At the time of the legislation, medical research was continually hampered by a shortage of animals for study. Oddly, the situation in New York City has not improved much despite the Metcalf-Hatch Law, simply because the institutes, hospitals and medical schools have not had the facilities necessary to procure and transport available animals from A.S.P.C.A. shelters to the laboratories. In New York City alone last year the A.S.P.C.A. destroyed 55,603 dogs and 102,658 cats; this is regrettable when one realizes the use to which many of these animals could have been put in research and teaching.
  4. The anti-vivisectionists have utilized the procurement and transportation difficulties that have faced medical science as a propaganda weapon. They have asserted that medical science has no need for these animals doomed to useless destruction. Nothing could be further from the truth. The A.S.P.C.A., despite severe anti-vivisectionist criticism, has complied to the letter of the law and has indicated its willingness to aid the New York State Society for Medical Research in its proposed transport function.

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5. To effect the maximum benefits of the Metcalf-Hatch Law and to overcome the procurement difficulties encountered by such New York City institutions as Rockefeller Institute, Sloan-Kettering Institute, Columbia University College of Physicians and Surgeons, New York University Medical College, Albert Einstein Medical School, Cornell University Medical College and the New York State School of Medicine and others, the New York State Society for Medical Research decided to assume the burden of activating an animal transport system. Accordingly, application was made to the United States Public Health Service for a grant to cover the purchase and maintenance of a vehicle designed specifically to transport animals. The United States Public Health Service saw fit to approve this request and such a truck has now been constructed. It is an ideal model of an animal transport facility. The body, on a  $1\frac{1}{2}$  ton chassis, has an aluminum exterior and a complete stainless steel interior with cages suited for dogs of varying size. Approximately 75 cats and 25 dogs can be carried comfortably. The interior is insulated, well illuminated and has arrangements for cooling, heating and drainage.

6. There is no doubt that the New York State Society for Medical Research faces a critical period. If its imminent venture fails, extremely powerful arguments would be placed in the hands of the fanatical but strong minority who clamor for repeal of the Metcalf-Hatch Law. Thus, these opponents of progress would cite medical science as disinterested in domestic animals as research material. If repeal occurs, the introduction into the State Legislature of anti-vivisectionist bills is certain. This latter would return us to the pre-Metcalf-Hatch period when these bills had to be vigorously opposed annually to preserve all animal experimentation. The New York State Society for Medical Research stands watch in this respect and is dedicated to the preservation of our existing enlightened legislation.

7. At this critical time in the New York State Society for Medical Research function, it is essential that its truly small assets be maintained. In the past, as now, the income of the Society derives chiefly from membership dues and sporadic contributory help. It now needs the solid backing that is given to medical research in other forms. It is our hope that by next year, the Society will not have to seek further foundation support. The projected plan for the coming year is clearly a pilot operation; its success will be a vital advance in medical research; its failure a serious setback, the repercussions of which would be felt on a national scale.

Specifically, these funds are requested to establish a full time Executive Secretary to relieve those research scientists who have thus far maintained and operated the Society on a voluntary basis. This full time officer is needed to:

- a. Keep contact with legislative activity in Albany.
- b. Oversee and direct a vigorous public education program on the importance and needs of medical research.
- c. Maintain public relations.
- d. Detect and counteract anti-vivisectionist attacks on medical research.
- e. Maintain membership at peak level by organizing and conducting the annual membership drive. (Membership has declined from 950 in 1951 to 600 in 1957.)
- f. Supervise the operation of the animal transport system.

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## 6. Budget Plan:

**Executive Secretary \$7,000**

Salaries

**Requested****\$5,000**

Expendable Supplies

Permanent Equipment

Overhead

Other

Total

**\$5,000**

## 7. Anticipated Duration of Work:

**One year for initiation of project (see above).**

## 8. Facilities and Staff Available:

**See above**

## 9. Additional Requirements:

**None**

## 10. Additional Information (Including relation of work to other projects and other sources of supply):

**None**

Signature

Director of Project

**Walter F. Riker, Jr.****Chairman, Animal Transport Committee**

Business Officer of the Institution

**V. E. Brooks****Treasurer**

1003540950

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10. Additional Information (including relation of work to other projects and other sources of supply):  
None

9. Additional Requirements: None

8. Facilities and Staff Available:  
See above

7. Estimated Duration of Work:  
One year for initiation of project (see above).

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JAN 7 1958

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RESEARCH & DEVELOPMENT  
ADPT.

6. Budget Plan:  
Executive Secretary \$1,000

Requested  
Salaries  
Expandable Supplies  
Permanent Equipment  
Overhead  
Other  
Total

\$2,000

\$2,000

Total



TIRC Grant #134R1

Tissue Culture Association  
Dr. Hans Ris, University of  
Wisconsin, Madison, Wisconsin

Final Report

**CONFIDENTIAL**

REPORT OF THE COURSE IN THE PRINCIPLES  
AND TECHNIQUES OF TISSUE CULTURE, 1960

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General

The Tissue Culture Association sponsored a summer course in "The Principles and Techniques of Tissue Culture" at the University of Wisconsin, Madison, Wisconsin, from June 20 to July 15, 1960. Transfer of the Course from Denver, where it was previously located, to Madison was carried out smoothly thanks to the cooperation of the faculty at Denver and the administration in Wisconsin. In particular, we were indebted to Dr. Hans Ris of the Department of Zoology in the University of Wisconsin, who has acted as our Sponsor this year, and who has been extremely helpful in assisting us in setting up the Course in new surroundings.

This year two major changes were introduced to the general arrangements. Whereas previously fifteen full-time students had been accepted and another fifteen places were filled by auditors, on this occasion all thirty participants were accepted as working members. This necessitated the purchase of a considerable amount of new apparatus and equipment and also placed more strain on the laboratory staff. In view of the increased number of working students, it may be desirable in future years to increase the laboratory staff. However, the experiment proved to be fully justified insofar as a more homogeneous teaching program became possible. The other major change related to the manner of staffing. In previous years, it had been customary to have the entire staff present throughout the Course. This year more Associates were invited to participate for shorter periods. Thus, instead of retaining three Associates for the whole Course, we had a total of eight Associates, two of whom were present at any time. This enabled us to present the students with a greater range of authoritative instruction and also to bring fresh enthusiasm into the teaching each week.

In other respects, the plan of instruction followed that established in previous years and constituted an intensive four-week course in the principles and techniques of tissue culture with particular emphasis on their application in research in fields of cell biology. The course of instruction included practice in all aspects of cell and tissue culture technique, and detailed consideration of the structure and function of living cells.

Morning sessions were devoted to lectures, discussions, demonstrations and practical laboratory exercises. As in 1959, these were designed as experiments to illustrate principles of cell biology. In the afternoons, opportunities were available to participate in research projects, special studies such as time-lapse cinemicrography, optical methods, and conferences with staff and visiting scientists. The evening lectures, Monday through Friday, were presented by members of the faculty and by a number of distinguished guest lecturers, selected to cover most aspects of current research in cell biology. Copies of the laboratory schedule and a list of evening lectures are appended.

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During the first week of the Course, emphasis was placed on basic methods for tissue and organ culture, especially in morphology and experimental embryology. The exercises included primary explanation techniques, organ cultures, disaggregation and reaggregation techniques, and whole embryo culture. During this week, Dr. Margaret Murray and Dr. Tom Algard were present as Associates in charge of teaching special subjects. In the second week, cell culture techniques were taught. These included methods for handling cell strains and methods for using them in quantitative studies. At the same time, instruction in the application of cell cultures to cytogenetic studies and virology were emphasized. Dr. Frank Ruddle and Dr. Calderon Howe were present as Associates during this week and took charge of instruction in their own special fields. During the third week, emphasis was placed on analytical methods applicable to tissue culture and, in particular, histochemistry and biochemistry. Dr. Hewson Swift conducted instruction in histochemistry, while Dr. William Rutter supervised instruction in biochemical methods of analysis. The final week of the Course dealt mainly with organizational problems and with the individual student's own research projects. Dr. Philip Marcus joined the staff during this time to participate in these discussions.

### Personnel

Lists of staff members and visiting lecturers, including their institutional connections, are appended.

We were fortunate in having the enthusiastic cooperation of a great many members of the University staff, in addition to our own personnel. In particular, Dean Robert Doremus extended help and advice which were greatly valued. Dr. Hans Ris, of the Department of Zoology, was the official Sponsor of the Course and provided liaison with the University administration. Dr. Ris also made arrangements for our accommodations and helped us in a great many ways. His enthusiastic support of this project contributed greatly to its success. We are also grateful to Dr. Robert Auerbach of the Zoology Department, who placed incubator facilities at our disposal. Throughout the Course we encouraged cooperation with the staff of the University of Wisconsin and the McArdle Laboratories and had a very friendly and profitable relationship with them. Members of the University staff who joined in our discussions included Dr. Ris, Dr. Van Potter, Dr. Lardy, Dr. Auerbach, Dr. Halvorson, Dr. Szybalski, Dr. Patau, and Dr. Swim.

The selection and recruitment of students was again in the hands of Dr. Mary Parshley, with the aid of Dr. Margaret Murray and Dr. Calderon Howe of the College of Physicians and Surgeons, New York. Once more about one hundred and fifty letters of inquiry were handled and between seventy and eighty applications were submitted. Dr. Parshley has informed us that already a number of applications have been received for next year's Course.

### Students

A list of participants is appended. These came from sixteen different states, and there were, in addition, three students from Great Britain, one from Switzerland, and one from Australia. The primary interests of the group included microbiology and virology (11), biochemistry (6), embryology and morphogenesis (6), pathology and cancer (4), endocrinology (1), radiobiology (1), and genetics (1). This distribution was very similar to that in 1959, but it was apparent this year that the divisions between disciplines were becoming much less sharp. Many of the microbiologists and embryologists were in fact primarily interested in biochemical aspects of their fields, while in cancer research the role of viruses has become especially important within the past two years.

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### Future Plans

During the Course its future was discussed by the Executive Council of the Tissue Culture Association. In view of the continued demand, in addition to the warm reception and evident appreciation of our activities by faculty members of the University of Wisconsin, it was decided that the Tissue Culture Association should continue to sponsor this project for a further period of five years and that an agreement with the University of Wisconsin for that period should be sought.

### Summary

The Tissue Culture Course was this year transferred from Denver to the University of Wisconsin and benefited greatly by the improved facilities thereby made available. It was successful in its aim of providing post-graduate education in cell biology by means of instruction by a highly qualified staff and a distinguished group of guest lecturers. The participants were once more of unusually high quality. Almost three times as many applications were received as places were available. In view of the continued demand, the Tissue Culture Association Executive Council has decided that this training program should be continued for a further five years beyond the expiry of the present period.

### FINANCIAL REPORT

Funds were available from collection of tuition fees (\$100 per student) and from specific grants. An outright grant of \$1,900.00 was received from the Tobacco Industry Research Committee for the purchase of two large incubators at \$950.00 each.

It is my great pleasure to express to the Tobacco Industry Research Committee the deep appreciation of the Tissue Culture Association, the staff, and the students for its interest and the financial assistance that has been provided in this teaching program to investigators in cell biology.

Respectfully submitted

/s/ John Paul

John Paul, Director  
Tissue Culture Course

1003540954

METHODS AND PRINCIPLES OF TISSUE CULTURE - STAFF - 1960

---

Director

Dr. John Paul  
HERT Tissue Culture Laboratory  
University of Glasgow  
Glasgow, Scotland

Assistant Director

Dr. William G. Cooper  
Department of Anatomy  
University of Colorado Medical School  
Denver, Colorado

Associates

First Week

Dr. Margaret R. Murray  
Laboratory for Cell Physiology  
College of Physicians and Surgeons  
New York, New York

Dr. Thomas Algard  
Department of Anatomy  
Stanford University  
Stanford, California

Second Week

Dr. Calderon Howe  
Department of Microbiology  
College of Physicians and Surgeons  
New York City, New York

Dr. Frank Ruddle  
Department of Zoology  
University of California  
Berkeley, California

Third Week

Dr. William J. Rutter  
Department of Chemistry and  
Chemical Engineering  
University of Illinois  
Urbana, Illinois

Dr. Hewson Swift  
Department of Zoology  
University of Chicago  
Chicago, Illinois

Fourth Week

Dr. George O. Gey  
Department of Surgery  
The Johns Hopkins Hospital  
Baltimore, Maryland

Dr. Philip I. Marcus  
Department of Biophysics  
University of Colorado Medical School  
Denver, Colorado

Laboratory Assistants

Miss Marilyn Bozeman  
Department of Virus and Rickettsial Diseases  
Walter Reed Army Medical Center  
Washington, D.C.

Mrs. Helena Benites  
Laboratory for Cell Physiology  
College of Physicians and Surgeons  
New York City, New York

Miss I Withers  
Department of Chemistry and Chemical  
Engineering  
University of Illinois  
Urbana, Illinois

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Photographic Analyst

Mr. John D. Carnes  
M.D. Anderson Hospital  
Texas Medical Center  
Houston, Texas

Service Assistants

Miss Jane Taplick  
Mr. John Merriam

Secretary

Mrs. Sarah Hughes

Sponsor and Adviser at the University of Wisconsin

Dr. Hans Ris, Department of Zoology

1003540956

## METHODS AND PRINCIPLES OF TISSUE CULTURE

### PARTICIPANTS - 1960 -

---

Allan R. Beaudoin, Ph.D., University of Florida, Gainesville, Fla.  
Dieter M. Burger, D.V.M., Washington State College of Veterinary Medicine, Pullman, Washington  
Richard P. Bunge, M.D., University of Wisconsin, Madison, Wis.  
Shu-Sing Cheng, Ph.D., University of North Carolina, Chapel Hill, N.C.  
Lionel Crawford, Ph.D., California Institute of Technology, Pasadena, Cal.  
William J. Culley, Ph.D., Muscatatuck State School, Indiana  
Donald P. Durand, Ph.D., University of Missouri, Columbia, Mo.  
Thomas Elsdale, Ph.D., University of Indiana, Bloomington, Indiana  
James L. German, M.D., Rockefeller Institute, New York City, N.Y.  
Robert E. Greefield, M.D., National Cancer Institute, Washington, D.C.  
Robert Hadek, Ph.D., D.V.M., University of Chicago, Chicago, Ill.  
R. H. Johnson, Dip. Bact., V.V.Sc., Veterinary Service, Nigeria  
Igor Klatzo, M.D., National Institute for Neurological Diseases and Blindness, Washington, D.C.  
Norman W. Klein, Ph.D., University of Connecticut, Storrs, Conn.  
Dolores P. Lawlor, A.B., Delafield Hospital, New York City, N.Y.  
Hans Loeffler, M.D., Children's Hospital, Philadelphia, Pa.  
Joseph P. Lowenthal, D.Sc., Walter Reed Hospital, Washington, D.C.  
Arlan McClurkin, Ph.D., U.S. Department of Agriculture, Iowa  
Joseph H. McSweeney, Ph.D., Fordham University, New York City, N.Y.  
Ruth C. Moore, M.Sc., Cancer Institute, Melbourne, Australia  
Erich D. Ryll, M.D., M.A. Bact., Burke Army Hospital, San Antonio, Texas  
William B. Savchuck, M.D., D.D.S., Pure Food and Drug Administration, Washington, D.C.  
John J. Sciarra, M.D., College of Physicians and Surgeons, New York City, N.Y.  
Vinod C. Shah, M.S., Columbia University, New York City, N.Y.  
Eric J. Simon, Ph.D., New York University, Bellevue, New York City, N.Y.  
Christopher P. Sword, Ph.D., University of Kansas, Lawrence, Kansas  
Al Szulman, Ph.D., Boston Lying-In Hospital, Boston, Massachusetts  
Harold M. M. Tovell, M.D., College of Physicians and Surgeons, New York City, N.Y.  
Clarence J. Weinman, Ph.D., University of Florida, Gainesville, Fla.  
Pauline J. Wood, Ph.D., University of Michigan, Ann Arbor, Mich.

1003540957

PROGRAM OF ACTIVITIES

1960

EVENING LECTURES

June 20	Dr. John Paul, Dept. of Biochemistry University of Glasgow, Scotland.	The cell and its environment.
June 21	Dr. Keith Porter, The Rockefeller Institute, New York City.	Cell ultrastructure.
* June 22	Dr. T. Algard, Dept. of Anatomy, Stanford University.	Some aspects of hormone action in vitro.
June 22	Dr. C. M. Pomerat, Dept. of Anatomy, University of Texas.	Dynamic morphology.
June 23	Dr. D. M. Prescott, Oak Ridge National Laboratory.	The growth cycle.
+ June 24	Dr. Honor B. Fell, Strangeways Laboratory, Cambridge, England.	Organ culture studies.
June 27	Dr. Margaret R. Murray, Laboratory for Cell Physiology, College of Physicians and Surgeons, New York City	Some problems of the nervous system approached through differentiating cultures.
June 28	Dr. George Yerganian, Children's Cancer Research Foundation, Inc., Boston.	Chromosomes of normal and malignant cells of the Chinese hamster.
June 29	Dr. F. Ruddle, Dept. of Zoology, University of California, Berkeley.	Chromosome variation in cell populations.
June 30	Dr. I. Lieberman, Dept. of Microbiology, University of Pittsburgh	Some aspects of cell nutrition.
* July 1	Dr. C. Howe, Dept. of Microbiology, College of Physicians and Surgeons, New York City	The erythrocyte as a reagent.
July 5	Dr. Hewson Swift, Dept. of Zoology, University of Chicago.	Cytochemical studies on giant chromosomes.
July 6	Dr. Richard A. Yates, E.I. DuPont de Nemours & Co., Wilmington, Delaware.	Feedback control of cell metabolism.
July 7	Dr. W. J. Rutter, Dept. of Chemistry and Chemical Engineering, University of Illinois	The metabolism of cell cultures.
* July 8	Dr. J. N. Davidson, Dept. of Biochemistry, University of Glasgow, Scotland.	Some biochemical aspects of growth.

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Program of Activities-Evening Lectures - 1960

+ July 11 Dr. H. Toolan, Sloan-Kettering Institute,  
New York City.

Studies on tissue  
transplantation.

July 12 Dr. P. Marcus, Dept. of Biophysics,  
University of Colorado Medical School.

The nature of viruses.

July 13 Dr. William G. Cooper, Dept. of Anatomy,  
University of Colorado Medical School.

Some aspects of the  
behavior of muscle  
tissue in culture.

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Meeting   Date

- |       |        |  |
|-------|--------|--|
| 9     | 29     | Discussion: Aspects of Variation in Cell Populations.  |
|       |        | Lab: Chromosome spreading and staining (Exp. 10).<br>Fix Maximow slide preparations (Exp. 2).  |
|       |        | Afternoon: Time-lapse cinemicrography.   |
| <hr/> |        |  |
| 10    | 30     | Discussion: Tissue Culture in Virology-I.  |
|       |        | Lab: Stain phytohemagglutinin cultures (Exp. 7).<br>Determine mitotic index (Exp. 2).  |
|       |        | Afternoon: Time-lapse cinemicrography.   |
| <hr/> |        |  |
| 11    | July 1 | Discussion: Tissue Culture in Virology-II.   |
|       |        | Lab: Virus hemagglutination and hemadsorption (Exp. 9).<br>Dulbecco plaque titration (Exp. 11).  |
|       |        | Afternoon: Time-lapse cinemicrography.   |
| <hr/> |        |  |
| 12    | 5      | Discussion: Histochemical methods.   |
|       |        | Lab: Set up test-tube cultures of L cells (Exp. 12).<br>Set up explants of chick mesonephros (Exp. 13).                                  |
| <hr/> |        |  |
| 13    | 6      | Discussion: Cytochemical methods.  |
|       |        | Lab: Section A - Metabolic experiment (Exp. 12).<br>Section B - Histochemistry (Exp. 13).<br>Section C - Tissue fractionation (Exp. 14). |
| <hr/> |        |  |
| 14    | 7      | Discussion: Control of cell metabolism.  |
|       |        | Lab: Section A - Histochemistry (Exp. 13).<br>Section B - Tissue fractionation (Exp. 14).<br>Section C - Metabolic experiment (Exp. 12). |
| <hr/> |        |  |
| 15    | 8      | Discussion: Nucleocytoplasmic relationships.   |
|       |        | Lab: Section A - Tissue fractionation (Exp. 14).<br>Section B - Metabolic experiment (Exp. 12).<br>Section C - Histochemistry (Exp. 13). |
| <hr/> |        |  |
| 16    | 9      | Discussion: Differentiation and adaptation to environment.<br>Review experiments.  |
|       |        | Lab: Examine and stain clones (Exp. 8).  |
| <hr/> |        |  |
| 17    | 11     | Discussion: Laboratory organization.<br>Sterilization materials and apparatus.   |

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<u>Meeting</u>	<u>Date</u>	
18	12	Discussion group: Morphology and differentiation.
19	13	Discussion group: Microbiology.
		Afternoon: Movies.
20	14	Discussion group: Biochemistry, etc.
		Evening: Graduation.

1003540962

TOBACCO INDUSTRY RESEARCH COMMITTEE  
350 FIFTH AVENUE NEW YORK 1, N. Y.

Salaries  
Expendable Services  
Application For Research Grant

Overhead 65  
Other Travel

Date: Form: 1-100-100-100

April 29, 1955

1. Name of Investigator: of Work: Two years  
Sydney C. Rittenberg

2. Title: and Staff Available: Rittenberg  
Professor of Bacteriology

3. Institution: No "State" beyond the present. *AVAILABLE*  
& Address: University of Southern California, Los Angeles 7, California  
Applicant for a graduate student, for 2/3 time.

4. Project or Subject:

Studies on the mechanism of bacterial metabolism of nicotine and related compounds. The ultimate goal of the project is the elucidation of the intermediary metabolism of nicotine oxidation.

5. Detailed Plan of Procedure (Use reverse side if additional space is needed; or sources of supply).

Although it is known that approximately 50% of the nicotine is oxidized in the body, the method of approach would consist of at least a four-phase attack employing, initially, procedures commonly used in metabolic studies.

The first phase would involve a study of the various environmental factors which influence nicotine oxidation by the particular bacterium used (under growing conditions), including the relation to the degree of aerobiosis, the pH, temperature, trace metal balances, and growth factor requirements. The organism would be subjected to variations of one factor, while keeping the others constant and both the extent of growth and the degree of nicotine utilization would be measured. Growth would be determined turbidimetrically, and the nicotine content would be determined chemically by available procedures. The oxidation of nicotine involves a *PHASE* the pyridine ring between the nitrogen and the carbonyl group. The second phase will consist of an attempt to uncover clues to the metabolic pathway by use of the technique of simultaneous adaptation (Stanier, 1947). Resting cells of the organism (adapted and unadapted to nicotine) will be presented with suspected intermediates, and the adaptive patterns which result will be correlated with a possible metabolic pathway. These studies would be extended to dried cells in order to eliminate permeability as a limiting factor.

*THE* The third phase would involve the isolation of intermediates. A two-fold approach will be employed: various metabolic inhibitors will be used, ranging from substances known to inactivate enzyme systems to anti-metabolites, which by virtue of their ability to compete with the substrate for the active sites on the enzyme, would cause an accumulation of intermediates; the second method of attacking this problem would be to subject cells to various growth conditions

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### Additional Information (Cont'd.)

From Preliminary studies in our laboratory, using a soil bacterium capable of utilizing nicotine as the sole source of carbon and nitrogen, have indicated that the pyridine ring is oxidized during the oxidation of nicotine. The evidence is based upon the amount of oxygen uptake observed during nicotine oxidation; it has consistently been in excess of that required for the oxidation of the pyrrolidine portion of nicotine. The organism employed appears to be similar to those previously reported in the literature (Bucherer, 1942, 1943; Choman et al. 1954; Sguros, 1954; Wada and Yamashita, 1954) in a number of respects. A blue pigment is produced during oxidation; the oxidation is extremely rapid; morphologically, the organism, a yellow chromogen, appears to be a gram negative rod containing gram positive granules. Using simultaneous adaptation as an analytical tool, preliminary observations have indicated that nicotine acid and pyridine, as such, are not metabolic intermediates. Resting cells were unable to utilize either of these compounds; dried cells also showed the same pattern.

Basic knowledge elucidating the pathway (or pathways) of nicotine oxidation would be valuable from several viewpoints. First, it would clarify the mechanism whereby a complex ring structure is handled. Secondly, it would enable one to determine how the utilization of the pyrrolidine and pyridine rings compares with the utilization of other cyclic compounds. Thirdly, since the quality of tobacco leaves is, in part, determined by their nicotine content, and the latter is greatly influenced by the microbial fermentations which occur during curing, it would be of interest to understand the chemistry of the micro-biological processes which occur during curing. Finally, although it does not necessarily follow that the mode of nicotine utilization by bacteria is similar to that in mammalian systems, past experience has indicated that in general, there does exist a great similarity in metabolic pathways among living forms. We see no reason for excluding nicotine from this general picture of Comparative Biochemistry.

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### Detailed Plan of Procedure (Cont'd)

The isolation of these intermediates would be accomplished by the use of chromatographic techniques as well as <sup>by</sup> the chemical isolation of intermediates.

The fourth phase will be on the cell-free level. Cells will be disrupted by various techniques (grinding, ultra-sonic treatment, freeze-thawing, and autolysis) in order to obtain nicotine oxidation by cell-free systems. If these attempts are successful, then fractionation of the enzyme system into its component parts becomes possible with the final characterization and confirmation of each step involved in the oxidation of nicotine.

The methodology, previously outlined, has proven to be successful in the characterization of other oxidative pathways, and no difficulties are anticipated except perhaps in the procurement of syntheses of possible intermediates.

1003540965

Source: <https://www.industrydocuments.ucsf.edu/docs/mnpl0000>

Application For Research Grant

Date:

July 15, 1957

1. Name of Investigator:

Sydney C. Rittenberg, *M.D.*

2. Title:

Professor of Bacteriology

3. Institution

& Address:

University of Southern California, Los Angeles 7, California.

4. Project or Subject:

The bacterial degradation of nicotine and related compounds.  
Studies on the fate of the products of nicotine degradation.

5. Detailed Plan of Procedure (Use reverse side if additional space is needed):

The plan of procedure to be employed in the proposed research program will be essentially the same as the one applied to the investigations now in progress. The successful application of the classical methods of enzymology to the elucidation of the primary step of nicotine oxidation makes it likely that a similar approach will be successful in working out the subsequent steps.

As was mentioned in the first annual report, a crude cell-free system has been obtained that carries nicotine oxidation to the  $3\mu\text{M}$  level (i.e., to a product accumulating after  $3\mu\text{M}$  of oxygen have been consumed per  $\mu\text{M}$  of nicotine). The crude extract has been partially fractionated to give preparations that cause the accumulation of the first and second oxidation products and the first product has been isolated and tentatively identified. We have indications that the second product can be isolated in a manner similar to the first, that is by precipitation with silicotungstic acid followed by ion-exchange chromatography using increasing concentrations of potassium chloride as the eluting agent. The next goal we have set is the isolation and identification of the second product and this may be achieved before the termination of the current grant.

The *enzymic* properties of the available enzyme fractions are such that it is certain we can cause the accumulation of the  $3\mu\text{M}$  oxidation product and almost certain that the same can be done for the  $1.5\mu\text{M}$  product without further fractionation procedures. Following the identification of the second step product we propose to go after these two products next. It is not certain at this time whether the same methods of isolation now employed for product one and two will be suitable for subsequent products since obviously the chemistry of each succeeding product will differ; consequently the methods to be employed will have to be chosen or developed as we go along.

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### 5. Detailed Plan of Procedure. (Cont'd)

For the fractionation of the crude enzyme system, we have only employed a single method so far (ammonium sulfate fractionation) and even this procedure has not been studied exhaustively. Consequently, after the products now available have been obtained further fractionations of the crude extract will be attempted using not only ammonium sulfate precipitation but also other procedures already available for this purpose. With complete success the first six oxidation products could be obtained; it is obviously impossible however to predict how successful we shall be.

In addition to the products now available by use of the enzyme preparation, the 7 $\mu$ m product (s) are also available by means of dry cell oxidation. Since this product (s) results from an extensive breakdown of the nicotine molecule with a release of about 50 percent of the initial carbon as CO<sub>2</sub>, it (they) should be a relatively small molecule and its identification by paper chromatography will be attempted.

It should also be mentioned that enzymes capable of carrying nicotine oxidation to levels between the 3 and 7 $\mu$ m stage might be obtainable from the dry cells or from fresh cells using different methods of extraction. Depending on progress along the avenues now open, attempts may be made to obtain the intermediate enzymes and thus make possible the isolation and identification of additional molecules in the oxidation sequence.

The final logical step would be the reconstruction of a complete cell-free system capable of oxidizing nicotine or any of the intermediates to the terminal stage of nicotine degradation. We are not so sagacious as to imagine this could be achieved within the next year but in view of the success that the enzymological approach has had in the current program continued progress is anticipated.

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6. Budget Plan:

Salaries	
Expendable Supplies	5,000
Permanent Equipment	1,500
Overhead	-
Other (8%)	511
(Travel)	300
Total	7,311

7. Anticipated Duration of Work:

1 year

8. Facilities and Staff Available:

All the equipment required for the investigations is available in our laboratory. No "staff" beyond the project director and a post doctoral fellow is required. The salary item in the budget is for a post doctoral fellow at full time.

9. Additional Requirements:

None.

10. Additional Information (Including relation of work to other projects and other sources of supply):

The relation of this problem to investigations of other contemporary workers is difficult to assess at the present time. To date, no investigations dealing with the intermediary metabolism of nicotine at the enzymatic level have been reported in the literature. This seems strange and such investigations would be in order in view of the increased proportion of the population coming into contact with nicotine. Certainly its metabolic fate poses a problem possessing more than an academic interest.

On the other hand, a number of papers have appeared dealing with what may be termed the "gross metabolism" of nicotine. These papers may be divided into two classes; mixed culture studies, and growth studies employing pure cultures. The methodology common to both these approaches has been the isolation of substances arising during growth at the expense of nicotine. From the chemical nature of these compounds, pathways were constructed based upon a logical sequence as determined by the structures of the substances. These results, valuable as they are, do not in themselves give any clue as to whether these compounds represent major pathways of degradation or are (see following page)

Signature \_\_\_\_\_

Director/Principal Investigator **Sydney C. Rittenberg**

Business/Office Manager **Robert D. Fisher**

Financial Vice-President

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10. Additional Information  
and other sources of

"metabolic dead ends." Nor do  
we determine which metabolic s.

The value of a research p  
in this laboratory, has been enu  
request; no reason to change these  
at the present time.

1003540970

**CONFIDENTIAL**

TIRC Grant #86AR1

Report No. 5

Sydney C. Rittenberg, Ph.D.  
University of Southern California

October 1957 - September 1958

Studies on the Mechanism of the Bacterial  
Metabolism of Nicotine and Related Compounds

Parts of the investigations conducted during 1957-58 have been described in the semi-annual report of June 9, 1958 and in two papers which will appear in the Journal of Biological Chemistry in January 1959. In addition, two papers are being prepared covering other aspects of the year's work. Consequently, this report will be brief, and the above mentioned semi-annual report and papers should be considered part of this annual report.

During the last half-year's work, investigations have yielded further information on the properties of the hydroxylating enzyme, the second oxidative product, and the blue pigment. These topics will be dealt with in turn.

Specificity of the hydroxylating enzyme

It had previously been shown that an enzyme fraction which precipitates from a crude extract between 20 and 40 percent ammonium sulfate saturation, hydroxylates and oxidizes nicotine to 6-hydroxynicotine. The same fraction was checked for its activity on a variety of compounds structurally related to nicotine. Pyridine, nicotinic acid, nicotinamide, anabesine, and 6-hydroxy-3-succinoylpyridine were not oxidized, but nor-nicotine and myosmine were oxidized with the consumption of 0.5 micromole of oxygen per micromole of substrate. With myosmine, the ultraviolet absorption maxima at 223 and 264 millimicrons changed to a single maximum at 298 millimicrons as a result of oxidation. The latter absorption maximum is characteristic of 6-hydroxymyosmine, thus leading to the conclusion that this compound was formed during the single step enzymatic oxidation. After the oxidation of nornicotine, the absorption maximum at 260 millimicrons disappeared and two new maxima at 232 and 300 millimicrons appeared. This change in the absorption spectrum is parallel to what occurs in the oxidation of nicotine to 6-hydroxynicotine; the 260 millimicron peak disappearing and peaks at 232 and 295 millimicrons appearing. On the basis of the observed spectral changes it can be tentatively concluded that 6-hydroxynornicotine is the product of oxidation. However, in this instance there is no data available in the literature for comparative purposes.

Thus, the enzyme hydroxylates certain compounds closely related to nicotine at the 6 position of the pyridine ring. It was this finding

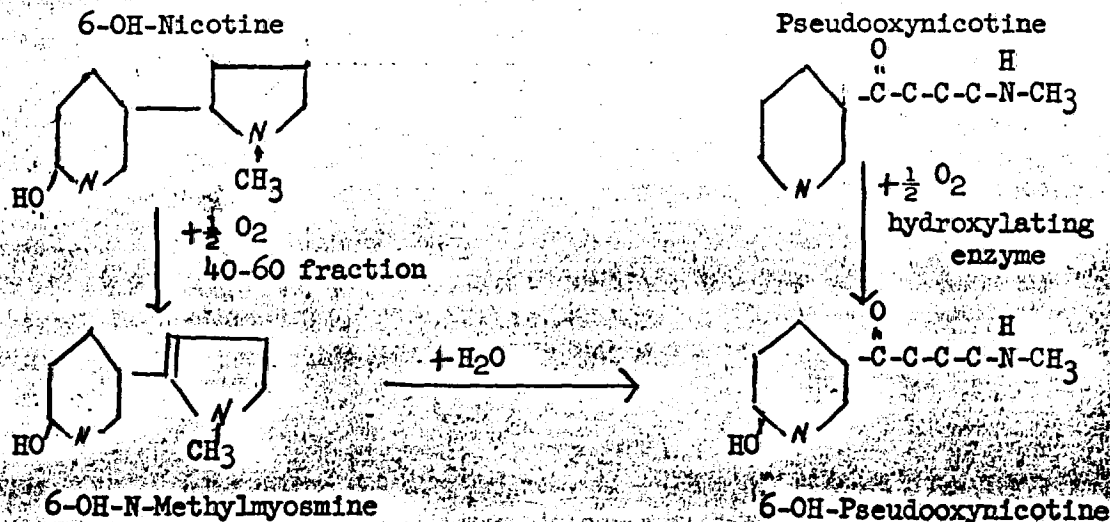
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that ultimately allowed a conclusive identification of the second oxidative product of nicotine (see below). Not too much can be said concerning the structural requirements of the enzyme. The hydroxylation of myosmine indicates that the N-methyl group of nicotine is not a necessary structural feature. Even though pseudooxynicotine is also oxidized (see below) this does not rule out the intact pyrrolidine ring as a requirement since ring closure could occur prior to oxidation. It is also apparent that this enzyme is not related to the hydroxylating enzyme involved in nicotinic acid oxidation, even though in the latter case it is also the six position of the pyridine ring that is attacked.

#### Identification of the second oxidative product of nicotine

Data presented in the semi-annual report (1958) suggested that the second oxidative product of nicotine degradation was 6-hydroxy-N-methylmyosmine. Elemental analyses of the enzymatically produced product indicated that it was isolated as a monohydrochloride salt. The product was metabolized by both resting cells and crude enzyme extracts in a manner consistent with its role as a true intermediate in nicotine degradation. Unfortunately, 6-hydroxy-N-methylmyosmine has not been described in the literature, and no information is available for comparative purposes. Thus a conclusive identification of the product by classical criteria is not immediately possible. Preliminary attempts to synthesize 6-hydroxy-N-methylmyosmine were unsuccessful; further efforts in this direction are presently underway. Fortunately, another approach to identification was possible.

Taking advantage of the spectrum of action of the hydroxylating enzyme, and its specificity for the six position, the oxidation of pseudooxynicotine by this enzyme was attempted. Oxidation occurred with the uptake of 0.5 micromole of oxygen per micromole of substrate, and yielded a product which, in the reaction mixture, had a single absorption peak at 290 millimicrons under acidic conditions. Under alkaline conditions, the peak shifted to a new maximum at 310 millimicrons, and the ratio of the two absorption maxima (alkaline/acid) was 2.16. The second oxidative product of nicotine degradation, formed by the single step oxidation of 6-hydroxynicotine, has identical absorption characteristics. Since it can be assumed, from the data presented in the previous section, that pseudooxynicotine was hydroxylated in the six position, the reactions with the two substrates can be formulated as follows:



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These data, combined with the previously available information establish 6-hydroxy-N-methylmyosmine (and/or its hydrated form, 6-hydroxypseudooxynicotine) as the second oxidative product in nicotine metabolism. There are some aspects of the chemistry involved that are still not clear. It is not known whether the hydrolytic step shown in the reaction sequence is an enzymatic one or not. It is possible that 6-hydroxy-N-methylmyosmine and 6-hydroxypseudooxynicotine are in spontaneous equilibrium under physiological conditions and that the acid base shift in ultraviolet absorption is due to the interconversion of the open and closed forms.

#### The blue pigment

If the 45-60 percent ammonium sulfate enzyme fraction is allowed to act on 6-hydroxymethylmyosmine in the presence of methylene blue or brilliant cresyl blue, oxygen is consumed and the substrate disappears. This disappearance is accompanied by the appearance of a new absorption maximum at 353 millimicrons. Under the same conditions, but in the absence of dye, no detectable oxygen uptake occurs, and a blue pigment that is apparently identical to the pigment formed during the growth of the bacterium on nicotine appears in the reaction mixture. No pigment is formed when the same reaction mixture is incubated under anaerobic conditions. Thus oxygen appears necessary for pigment formation although it does not appear to be consumed during the reaction.

In order to study this phenomenon further, an effort was made to obtain a quantitative measure of pigment formation. Using pure, crystalline 6-hydroxynicotine as the substrate and the 45-60 fraction as the enzymatic material, reactions were run in the absence of dye over a range of substrate concentrations between 0.01 and 0.04 micromoles per two ml. Production of pigment was followed spectrophotometrically by measuring absorbance at 600 millimicrons. When absorbance reached a maximum and then remained constant with time, it was assumed that the substrate had been completely consumed. This was confirmed by failure to detect 6-hydroxynicotine or 6-hydroxymethylmyosmine spectrophotometrically in such reaction mixtures. Assuming a quantitative conversion of substrate to pigment, a molar extinction coefficient for the latter was calculated and used to determine the amount of pigment formed in other experiments. On the basis of several such experiments, the following information was obtained:

(1) the pigment has a very high molar extinction coefficient, of the order of 5300. This means that very intensely colored solutions can result from minute quantities of pigment.

(2) the rate of pigment formation, under conditions where substrate was not completely consumed during the course of the experiment, decreased markedly with time. For example, starting with 2.6 micromoles of 6-hydroxy-methylmyosmine, only 0.36 micromole of pigment was formed in two hours using 2.5 mg. enzyme protein. From such results, it was apparent that conventional manometric techniques are not sufficiently sensitive to determine whether oxygen is consumed during pigment formation. If one

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assumes that one-half micromole of oxygen is taken up per micromole of substrate converted to pigment, then a total of only 4 microliters of oxygen would have been used under the conditions of the experiment described. This would not be detectable with the conventional Warburg apparatus and no instrument of greater sensitivity is available to us.

In another series of experiments, the rate of pigment formation was compared in reaction mixtures incubated anaerobically previous to exposure to air with mixtures shaken in air from the start. It was found that an anaerobic incubation resulted in a more rapid rate of pigment formation once air was introduced. Further, although no pigment was formed anaerobically, a new compound that differed from 6-hydroxymethylmyosmine and the third oxidative product of nicotine was detected chromatographically and spectrophotometrically. These data show that one or more intermediates exist between 6-hydroxymethylmyosmine and pigment, and are consistent with the previously postulated intermediate described as compound X in the semi-annual report. It is possible that this anaerobic step is the hydrolytic removal of methylamine from the molecule. This hypothesis is being investigated currently, concomitant with the studies on the third oxidative product.

Attempts to isolate the pigment in pure form have not succeeded. Analyses on the best two samples so far obtained showed that the product as isolated was grossly contaminated with inorganic materials. We are continuing our efforts to obtain a pure product.

Once formed the pigment was found to be metabolically inert. Thus when pigment formation from second product was allowed to occur in the absence of dye and then dye was tipped into the system to allow oxidation of residual substrate, the amount of pigment remaining at the end of oxidation was unaltered.

Speculations on the chemical nature of the pigment are premature and the complete elucidation of its structure is probably beyond the scope of this investigation. It does appear to us however that the pigment must be at least a dimer of some base unit derived from 6-hydroxymethylmyosmine since no simple alteration of the second product either by the addition of water or by the uptake of a small amount of oxygen can be conceived that would yield such an intensely pigmented compound. Our data do show clearly, however, two points of importance in the relation of pigment formation to nicotine metabolism: the pigment is formed between the second and third oxidative step; and that it is a side product and not an intermediate in nicotine degradation.

1003540974

Committee

Jacobson, Chairman  
Kotin  
Little

#209R2

Activated 10/1/58

Renewed 10/1/59

Cf. #86A

Activated 10/1/56

Renewed 10/1/57

TOBACCO INDUSTRY RESEARCH COMMITTEE  
150 East Forty Second Street New York 17, N.Y.

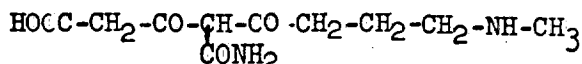
Application For Research Grant

Date: May 13, 1960

1. Name of Investigator: Sydney C. Rittenberg, Ph.D.
2. Title: Professor of Bacteriology
3. Institution & Address: University of Southern California  
Los Angeles 7, California
4. Project or Subject: The Bacterial Oxidation of Nicotine.
5. Detailed Plan of Procedure:

Our studies on the bacterial oxidation of nicotine have been supported by a series of grants from the TIRC since Oct. 1, 1956. The detailed plan of procedure and results obtained have been presented to the Committee in previous grant applications, in semiannual and annual reports, and in a series of articles in the Journal of Biological Chemistry. In general, we expect to continue our investigations along the lines previously followed. Specifically, during the remaining months of the current grant and in the following year, we expect (1) to complete the identification of the blue pigment, (2) to isolate and identify 4th and 5th product, and (3) to survey by the simultaneous adaptation technique a group of 10-15 nicotine-oxidizing bacteria to determine if the metabolic pathway of the bacterium we are studying is common to most or all nicotine-oxidizing bacteria. The background for the above goals was given in the last semi-annual report and will not be repeated here.

From the evidence already available it seems almost certain that both the pyridine and pyrrolidine rings have been opened by the end of the 5th step. A reasonable guess is that a molecule like the following exists at this stage:



The position chosen for the amide and carbonyl group on what was the pyridine ring may not be correct. However, it is clear that labile sites must exist and further oxidative steps should result in rapid fragmentation of the chain, forming small molecules like methylamine, malonate, succinate, acetate, butyrate and propionate. Since the metabolic pathways of such compounds are reasonably well known, our original goal would be achieved as soon as the fragments of further oxidation are identified as to nature and carbons of origin.

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Assuming the rate of progress during the first year is as estimated above, our second year's work would be concerned with the study of the "fragments." At this point it may prove necessary to work with C-14 labeled nicotine in order to pinpoint the fate of the individual carbons in the nicotine molecule. Since this could involve the synthesis of specifically labeled nicotine, the proposed materials budget for the second year has been increased. Having somewhat underestimated the time factor in previous grant requests, I hesitate to state where we should be at the end of the two years proposed. I feel however that we should be fairly near to the overall completion of the project.

6. Budget Plan:

	1st yr.	2nd yr.
Salaries	8,400.	3,600.
Expendable Supplies	1,000.	2,000.
Permanent Equipment	-	-
Overhead (8%)	780.	476.
Other (Travel)	350.	350.
Total	\$10,530.	\$6,426.

7. Anticipated Duration of Work:

Two years to complete the work outlined. Perhaps one additional year to complete the project as originally conceived.

8. Facilities and Staff Available:

Complete facilities available for work proposed.

Staff: Dr. S. C. Rittenberg, Professor of Bacteriology, Director  
Dr. S. H. Richardson, Research Associate, full time at \$6,000 per year. Dr. Richardson has worked on this project for two years and has just completed all requirements for his Ph.D. degree.

Mr. R. Gherna, Research Assistant, approximately half time at \$2,400 per year. Mr. Gherna has worked for two summers on this project as an undergraduate assistant. He will start work toward his M.A. degree in Sept. 1960.

9. Additional Requirements: None

10. Additional Information:

See previous grant applications and semiannual and annual reports.

Signature Sydney C. Rittenberg  
Director of Project

Business Officer of Institution

1003540976

# The Bacterial Oxidation of Nitrobenzene

Grants # 209 &  
86

THE BACTERIAL OXIDATION OF NITROBENZENE  
IDENTIFICATION AS A METABOLIC PRODUCT

L. J. HIGGINS AND G. W. H. HIGGINS

From the Department of Microbiology, University of Southern California, Los Angeles, California

(Received for publication, August 18, 1957)

in a total volume of 50 ml. The flask stood in a water bath at 30°C. and was shaken for 24 hr. The medium was then poured into a 100-ml. Erlenmeyer flask and allowed to stand at room temperature.

The medium was then poured into a 100-ml. Erlenmeyer flask and allowed to stand at room temperature. The medium was then poured into a 100-ml. Erlenmeyer flask and allowed to stand at room temperature. The medium was then poured into a 100-ml. Erlenmeyer flask and allowed to stand at room temperature.

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in a total volume of 50 ml. The flask stood in a water bath at 30°C. and was shaken for 24 hr. The medium was then poured into a 100-ml. Erlenmeyer flask and allowed to stand at room temperature. The medium was then poured into a 100-ml. Erlenmeyer flask and allowed to stand at room temperature. The medium was then poured into a 100-ml. Erlenmeyer flask and allowed to stand at room temperature.

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## The Bacterial Oxidation of Nicotine

### II. THE ISOLATION OF THE FIRST OXIDATIVE PRODUCT AND ITS IDENTIFICATION AS (1)-6-HYDROXYNICOTINE\*

L. I. HOCHSTEIN† AND SYDNEY C. RITTENBERG

*From the Department of Bacteriology, University of Southern California, Los Angeles, California*

(Received for publication, August 18, 1958)

It has been previously shown (1) that cell-free extracts from a soil bacterium, P-34, oxidize nicotine with the consumption of 0.5  $\mu$ mole of oxygen per  $\mu$ mole of nicotine, producing a compound which possesses a characteristic ultraviolet absorption spectrum. Preliminary data suggested that this compound was either a 2- or 6-pyridone derivative of nicotine. This paper presents evidence which indicates that this compound is the first oxidative product of nicotine degradation and is indeed (1)-6-hydroxynicotine (6-OHN).<sup>1</sup>

#### EXPERIMENTAL

The conditions of growth and the preparation and fractionation of cell-free extracts have been described previously (1).

2-OHN was synthesized from nicotine according to the methods of Chicababin and Kirssanow (2) and Wada (3). Samples of 2-OHN and 6-OHN were obtained as gifts through the generosity of Dr. E. Wada, Central Research Institute, Japan Monopoly Corporation, Tokyo, Japan.

Infrared spectra were determined with a Perkin-Elmer model 137 Infracord double beam spectrophotometer. The sample was made up in potassium bromide by mixing 1 part of sample with 100 parts of anhydrous potassium bromide. The mixture was agitated for 2 minutes in a Wig-L-Bug amalgamator,<sup>2</sup> and the resulting powder was compressed at 18,000 pounds/sq. in. in a vacuum.

Ultraviolet spectrophotometry, paper chromatography, and manometry were carried out as previously described (1). Melting point determinations were made in sealed capillary tubes and were uncorrected. Optical rotations were determined in a Rudolph High Precision Polarimeter, model 80.

#### RESULTS

*Isolation of First Oxidative Product from Enzymatic Reaction Mixtures*—2000  $\mu$ moles of nicotine (324 mg.), 10  $\mu$ moles of methylene blue, 490  $\mu$ moles of potassium phosphate buffer, pH 7, and the 20 to 40 fraction were incubated in an Erlenmeyer

flask in a total volume of 20 ml. The flask, attached to a mercury manometer, was placed on a rotary shaker and incubated at 30°.

When oxygen consumption approached 0.5  $\mu$ mole of oxygen per  $\mu$ mole of nicotine, 2 volumes of 0.1 N HCl were added to the reaction mixture and the precipitated protein was removed by centrifugation. The acidified solution was treated with an excess of Dowex 50 in the acid form until the supernatant solution failed to give a precipitate with STA. The resin was centrifuged, washed with 0.1 N HCl, and suspended in 0.1 M KCl. The resin was removed by centrifugation, the eluate was saved, and the resin was suspended in fresh 0.1 M KCl and again centrifuged. The combined eluates were evaporated to dryness at reduced pressure to yield a yellowish residue. This was exhaustively extracted with several portions of boiling Skellysolve B and the solvent fractions were combined and partially evaporated. Crystallization was permitted to occur at room temperature yielding an amorphous solid having a yellowish tinge. A second crystallization yielded a material which melted at 109–119°. After treatment with Norit A, recrystallization from boiling Skellysolve B yielded a white crystalline material which melted at 120–122° (yield, 144 mg.).

*Isolation of First Product from Growth Medium*—During growth of P-34 in a nicotine-yeast extract-mineral salts medium, a product accumulated whose absorption spectrum was similar to the substance produced by the 20 to 40 fraction. Preliminary studies indicated that the accumulation was maximum after 36 hours of growth and that it was accompanied by the formation of a greenish fluorescent pigment (4).

In order to isolate the product, 28 l. of the growth medium were distributed in 7-l. lots and incubated as previously described. After 36 hours of growth, the cells were collected in a Sharples centrifuge and the greenish supernatant fluid was acidified to pH 2 with concentrated HCl and treated with STA to precipitate the product. An aqueous neutral solution of the product was obtained from the STA salt by following the procedure of Frankenburg *et al.* (5). This solution was evaporated to dryness and treated with Skellysolve B as previously described. After 3 recrystallizations from Skellysolve B, 8 gm. of a white crystalline material were obtained (m.p. 121–121.5°).

*Metabolic Behavior of Product*—Nicotine-grown resting cells oxidized the isolated product and nicotine at the same rate (Fig. 1). At the cessation of oxidation the consumption of oxygen in

\* This work was supported by a grant from the Tobacco Industry Research Committee.

† Present address, Department of Medical Microbiology, University of Southern California School of Medicine, Los Angeles, California.

<sup>1</sup> The abbreviations used are: 2-OHN, 2-hydroxynicotine; 6-OHN, 6-hydroxynicotine; STA, silicotungstic acid.

<sup>2</sup> Manufactured by Crescent Dental Manufacturing Company, Chicago, Illinois.

the case of the product was 0.5  $\mu$ mole of oxygen per  $\mu$ mole of substrate less than in the case of nicotine.<sup>3</sup>

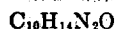
Crude extracts oxidized nicotine and the product at the same initial rate (Fig. 2). In the case of nicotine oxidation, the rate of oxygen uptake changed after the consumption of 1  $\mu$ mole of oxygen per  $\mu$ mole of nicotine. The rate of product oxidation changed after the consumption of 0.5  $\mu$ mole of oxygen per  $\mu$ mole of substrate. After these changes in rates, the rates of oxygen consumption for nicotine and product oxidation were essentially the same. The product was not oxidized by the 20 to 40 fraction which oxidized nicotine only to the 0.5  $\mu$ mole level (1). With the product as substrate, the 40 to 60 fraction (which oxidizes nicotine only to the 1  $\mu$ mole level) and the crude extract consumed the same amount of oxygen at the cessation of oxidation and at the first change in rate respectively (Table I).

The stoichiometry of oxygen consumption by resting cells as well as the stoichiometry and rate of oxygen consumption by various cell-free extracts indicate that the product is an intermediate in nicotine degradation and not a product of a side reaction.

**Properties of Product**—The products isolated from enzymatic reaction mixtures and growth medium were judged to be identical by the following criteria: identity of ultraviolet absorption spectra; similarity of melting points; no significant depression on mixed melting point determinations; and identical behavior during chromatography (Table II).

The product did not give rise to a chromogenic compound when treated with cyanogen bromide and  $\beta$ -naphthylamine according to the method of McCormick and Smith (6). The presence of a hydroxyl group was indicated by the formation of a deep burgundy color with ceric nitrate (7). Upon standing, the color faded. That this functional group was a pyridone seemed probable from the nature of the ferric chloride reaction. The product reacted with ferric chloride under acid conditions (in 0.2 N HCl), but not under neutral conditions, giving rise to an orange-red color, a behavior typical of pyridones (8).

An elemental analysis gave<sup>4</sup> the following results:



Calculated: C 67.42, H 7.86, N 15.73

Found: C 67.46, H 8.00, N 15.62

The product was optically active, exhibiting an  $(\alpha)_D$  of  $-54.8^\circ$  in aqueous solutions.

Although the melting point of the product was similar to the reported melting point of 2-OHN (9), comparison of the product with synthetic samples of 2-OHN indicated that the substances were not identical. The ultraviolet absorption spectra of 2-OHN and the product differed significantly. The absorption maxima of 2-OHN were located at 228 and 303  $m\mu$  while those of the product were located at 232 and 295  $m\mu$  (Fig. 3). Furthermore, the  $A_{232/295}$  for 2-OHN was considerably lower than that for the biological product. 2-OHN failed to give rise to chromogenic compounds when treated with either ceric nitrate or acidic ferric chloride. The picrate derivative of 2-OHN melted some  $30^\circ$  higher than did the corresponding derivative of

<sup>3</sup> The product was assumed to be a hydroxynicotine, molecular weight 178, for this calculation.

<sup>4</sup> The analysis was performed by Dr. A. Elek, Elek Micro Analytical Laboratories, Los Angeles, California.

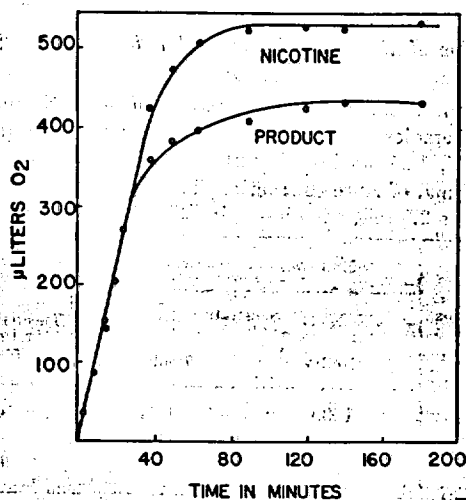


FIG. 1. The oxidation of nicotine and the first oxidative product by nicotine-grown resting cells. Experimental conditions: 3.8  $\mu$ moles of nicotine, 3.5  $\mu$ moles of product, 70  $\mu$ moles of potassium phosphate buffer, pH 7, 0.25 ml. of a resting cell suspension. Total volume 2.0 ml., gas phase air,  $30^\circ$ .

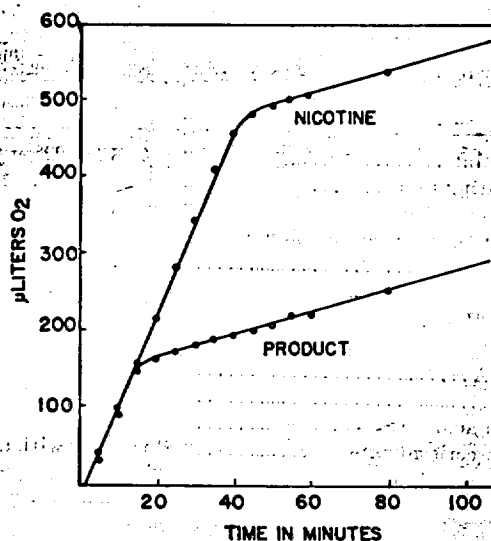


FIG. 2. The oxidation of nicotine and the first oxidative product by a crude extract. Experimental conditions: 20  $\mu$ moles of nicotine, 15  $\mu$ moles product, 102  $\mu$ moles of potassium phosphate buffer, pH 7, 1.25  $\mu$ moles of methylene blue, and 17.5 mg. of crude extract. Total volume, 2.0 ml., gas phase air,  $30^\circ$ .

the biological product (Table II). 2-OHN did not prove to be metabolically active. Crude extracts which oxidized nicotine beyond the oxidation level of the biological product did not oxidize 2-OHN. Finally, and most significantly, the infrared spectra of the two compounds were markedly different. The absorption spectrum of 2-OHN exhibited a pronounced band at approximately 13  $\mu$  whereas the absorption spectrum of the biological product lacked this feature (Fig. 4).

On the other hand, several properties exhibited by synthetic 6-OHN suggested that it might be identical to the metabolic product. Its ultraviolet absorption spectrum was essentially identical to that of the metabolic product (Fig. 3). The  $A_{232/295}$  of both compounds was in good agreement. Authentic 6-OHN reacted with ceric nitrate and ferric chloride in a manner identical

TABLE I

Oxidation of nicotine and first product by various cell-free extracts

Experimental conditions: 19  $\mu$ moles of 6-OHN (enzymatic product), 20  $\mu$ moles of nicotine, 102  $\mu$ moles of potassium phosphate buffer, pH 7, 1.25  $\mu$ moles of methylene blue, 17.5 mg. of crude extract, 7.5 mg. of 20 to 40 fraction, 14.5 mg. of 40 to 60 fraction. Total volume 2.0 ml., gas phase air, 30°.

Enzyme fraction	$\mu$ Moles of oxygen consumed per $\mu$ mole of substrate*		
	Substrate		Theoretical for the first oxidative product
	Nicotine	Product	
Crude	1.09	0.41	0.5
20 to 40	0.48	0	0
40 to 60	1.05	0.43	0.5

\* For the crude extract, the oxygen consumption reported is to the first change of rate; all other values represent oxygen consumption at the cessation of oxidation.

III). This would indicate that crude extracts contain an enzyme capable of racemizing 6-OHN.

At the cessation of oxidation of the biological product by the 40 to 60 fraction, the absorption spectrum of the reaction mixture changed. The absorption maximum at 232  $m\mu$  disappeared and the maximum at 295  $m\mu$  shifted to 290  $m\mu$ . Thus the change in absorption was similar to that observed in reaction mixtures in which crude extracts had oxidized nicotine with the consumption of 1  $\mu$ mole of oxygen per  $\mu$ mole of nicotine prior to the change in oxidative rate (1).

## DISCUSSION

The characterization of the first oxidative product of nicotine degradation by strain P-34 as (1)-6-OHN confirms, at least in part, the earlier report of Frankenburg and Vaitekunas (10) who isolated 6-OHN during the fermentation of nicotine by a tobacco seed infusion. The product isolated by these workers was apparently a racemic mixture as judged by the reported melting point of their compound and its failure to depress the melting

TABLE II

Properties of biological product and synthetic 2- and 6-OHN

Property	Biological products		Synthetic products	
	Growth medium	Enzymatic reaction	2-OHN	6-OHN
Melting point.....	120-122°	121.5-122°	121-123°	103-105°
Mixed melting point.....	119.5-121.5°			
Melting point of picrate.....	164.5-165°		196-198° (9)	221-222° (2)
Ultraviolet absorption maximum in 0.1 N HCl.....	232 $m\mu$	232 $m\mu$	228 $m\mu$	232 $m\mu$
	295 $m\mu$	295 $m\mu$	303 $m\mu$	295 $m\mu$
$\epsilon$ (in 0.1 N HCl).....	12250		7000	12300
	5750		7600	5650
$A_{222/295}$ .....	2.13	2.17	0.92	2.18
$R_F$ .....	0.12-0.13	0.13-0.15	0.19	
Color with acid ferric chloride.....	Orange-red	Orange-red	No reaction	Orange-red
Color with ceric nitrate.....	Burgundy with fading	Burgundy with fading	No reaction	Burgundy with fading

to the biological product. Furthermore, the infrared spectra of both compounds were in excellent agreement (Fig. 4). Finally, synthetic 6-OHN proved to be metabolically active. Nicotine-grown resting cells and crude extracts oxidized synthetic 6-OHN in a manner which suggested that it was indeed an intermediate of nicotine degradation.

However, synthetic 6-OHN and its picrate derivative possessed melting points which differed significantly from those of the biological product and its picrate. The melting points of the free bases differed by some 19° whereas the melting points of the picrate derivatives differed by some 60° (Table II). Since the synthesis of 6-OHN from nicotine leads to the formation of a racemic mixture (2), the disparity of the melting points was ascribed to the comparison of a racemic mixture with a levorotatory enantiomorph and thus was not considered significant.

With use of the 40 to 60 fraction, the net oxygen uptake with the racemic, synthetic product as the substrate was one-half of that with the biological product. However the crude extract oxidized both materials with the same oxygen uptake (Table

point of a synthetic sample of 6-OHN. Whether racemization occurred as a consequence of their isolation procedure or was due to enzymatic action is difficult to assess because of the complexity of the environmental conditions which were employed as well as the failure of these workers to describe their isolation procedure. In view of the isolation of 6-OHN and other compounds from fermentation mixtures, these authors postulated 3 pathways for the bacterial degradation of nicotine: the "pyridine pathway," in which 6-OHN is the first oxidative product; and the "pyrrolidine pathway," which in reality consisted of two pathways which diverged after the formation of  $\gamma$ -keto- $\gamma$ -(3-pyridyl) butyric acid, in which pseudo-oxynicotine was proposed as the first product.

Wada (3), who investigated the oxidation of nicotine by a group of soil bacteria, isolated and identified a series of compounds from the spent growth medium. He proposed that nicotine degradation is initiated at the pyrrolidine ring to yield *N*-methylmyosmine as its hydrated species, pseudo-oxynicotine. Some of the soil isolates were also capable of degrading nor-

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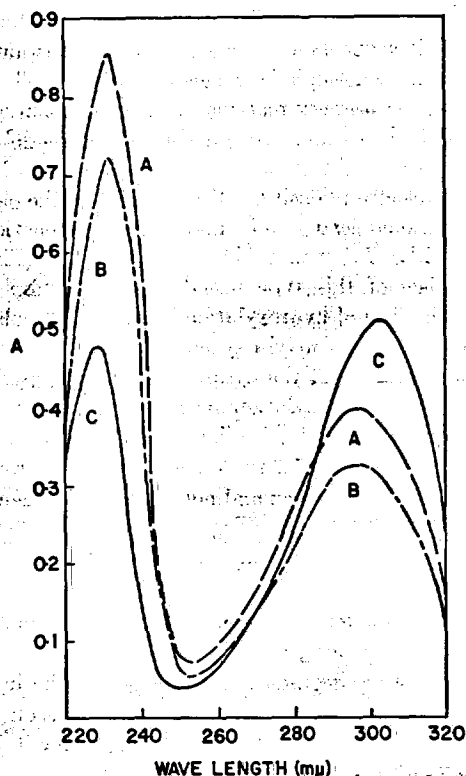


FIG. 3. The ultraviolet absorption spectra of the first oxidative product and synthetic 2-OHN and 6-OHN. A, first oxidative product (12.4  $\mu\text{g.}$ ); B, synthetic 6-OHN (10.4  $\mu\text{g.}$ ); C, synthetic 2-OHN (12.2  $\mu\text{g.}$ ). The absorption spectra was made in 0.1 N HCl.

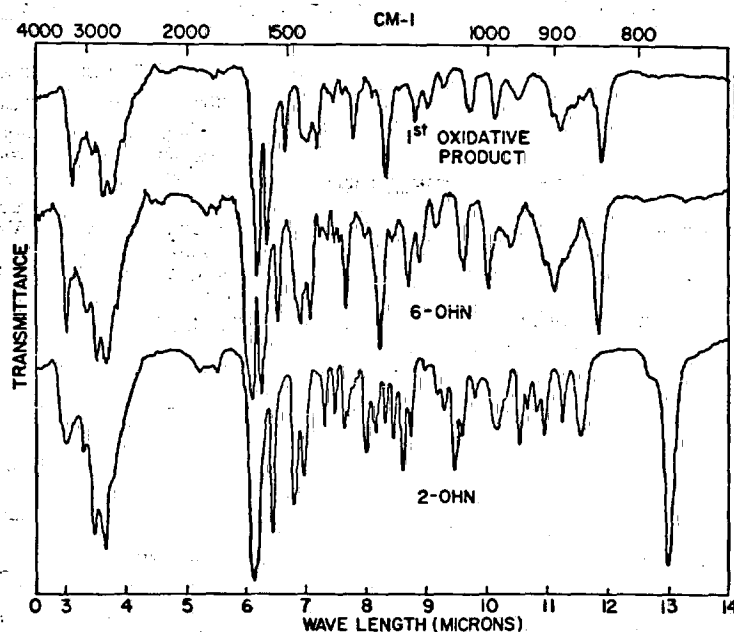


FIG. 4. The infrared spectra of the first oxidative product, synthetic 2-OHN, and synthetic 6-OHN.

nicotine, the demethylated analogue of nicotine. However, the metabolism of this compound was initiated by a hydroxylation reaction at the 6 position of the pyridine ring followed by a dehydrogenation of the pyrrolidine ring to yield 6-hydroxy-

myosmine. Preliminary evidence indicates that strain P-34 further degrades 6-OHN by a dehydrogenation of the pyrrolidine ring to yield 6-hydroxy-*N*-methylmyosmine, the methylated analogue of 6-hydroxymyosmine. Thus P-34, unlike the organisms studied by Wada, appears to degrade nicotine by a pathway similar to the pathway of nornicotine degradation suggested by Wada.

The degradation of nicotine by *Corynebacterium nicotinovorum* has been reported to yield *N*-methyl-2-(3-pyridyl)-1-pyrrolidium hydroxide and *N*-methyl-2-(3-pyridyl)-1,2-pyrrolidium hydroxide (11). The reported ultraviolet absorption spectrum of the former compound is in remarkable agreement with the spectrum exhibited by 6-OHN. The latter compound possesses an absorption spectrum essentially the same as the spectrum exhibited by our reaction mixtures after oxidation of nicotine to the 1  $\mu\text{mole}$  of oxygen level. As the proposed products of *C. nicotinovorum* metabolism were not isolated in a state which permitted an unequivocal determination of their chemical and physical properties, no direct comparison with our product is possible. However, the marked similarity of the ultraviolet absorption spectra, although not conclusive evidence (12), would suggest that a reinvestigation of these compounds would be in order to determine whether nicotine degradation by *C. nicotinovorum* occurs by a pathway similar to that observed in strain P-34 or whether a different pathway of nicotine degradation does indeed exist.

By virtue of the requirement for methylene blue, the reactions leading to the formation of 6-OHN must involve a dehydrogenation step. As the reaction is accompanied by the consumption of 0.5  $\mu\text{mole}$  of oxygen per  $\mu\text{mole}$  of nicotine, and the product contains 1 gm. atom of oxygen per mole, the product

oxygen must have its origin in water oxygen rather than molecular oxygen. Thus the over-all reaction can be conceived as being the sum of 2 reactions: one involving the addition of oxygen, the other the dehydrogenation step.

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TABLE III

Oxidation of synthetic 6-OHN by cell-free extracts

Experimental conditions: 19.3  $\mu$ moles of synthetic 6-OHN, 12  $\mu$ moles of biological product, 50  $\mu$ moles of potassium phosphate buffer, pH 7, 0.5 ml. of crude extract, 4.2 mg. of 40 to 60 fraction. Total volume 2.0 ml., gas phase air 30°.

Extract	$\mu$ Moles of oxygen per $\mu$ mole of substrate	
	Synthetic 6-OHN	Biological product
Crude*	0.56	0.5
40 to 60†	0.27	0.5

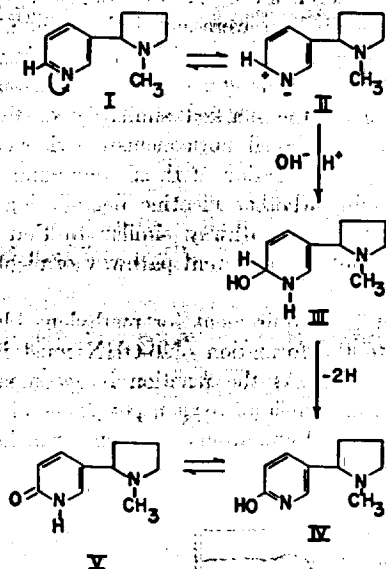
\*  $\mu$ Moles oxygen per  $\mu$ mole nicotine to the first rate change.†  $\mu$ Moles oxygen per  $\mu$ mole of 6-OHN at the cessation of oxidation.

Fig. 5. The proposed pathway for the formation of 6-OHN from nicotine.

Since carbon-6 of the pyridine ring is a center of low electron density (13), it would be expected to be subject to nucleophilic attack. If the nucleophilic reagent is conceived as being hydroxyl ion, the primary reaction in the conversion of nicotine (I) to 6-OHN (IV) could proceed through a pseudobase (III) via a resonance hybrid of nicotine (II) in which carbon-6 is a center of low electron density. The addition of the elements of water to II would lead to III and a dehydrogenation of III would yield either IV or its pyridone tautomer (V) (Fig. 5).

A mechanism of this type would be a departure from the classical mechanism of hydroxylation in which molecular oxygen is the source of the hydroxyl oxygen found in the product (14). However, it may be that the hydroxylation of the pyridine ring is unique among hydroxylation reactions, for in the bacterial oxidation of nicotinic acid, leading to the formation of 6-hydroxynicotinic acid (15-17), the oxygen of the hydroxyl group has its origin in water oxygen and not molecular oxygen (15, 18).

## SUMMARY

A compound, isolated from enzymatic reaction mixtures and from the growth medium, was shown to be the first oxidative product of nicotine degradation by a soil bacterium. This compound was identified as (1)-6-hydroxynicotine on the basis of its correspondence in properties, including infrared spectrum, with synthetic 6-hydroxynicotine.

A tentative reaction mechanism has been proposed in which the first step is the formation of a pseudobase by the addition of water across carbon-6 and the nitrogen of the pyridine moiety of nicotine, followed by the oxidation of the pseudo-base to (1)-6-hydroxynicotine, or its pyridone tautomer, by a methylene blue-dependent step.

**Acknowledgment**—The authors are grateful to Dr. Jerome A. Berson, Department of Chemistry, University of Southern California, for his advice in the field of heterocyclic chemistry.

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# The Bacterial Oxidation of Nicotine

## I. NICOTINE OXIDATION BY CELL-FREE PREPARATIONS\*

L. I. HOCHSTEIN† AND SYDNEY C. RITTENBERG

*From the Department of Bacteriology, University of Southern California, Los Angeles, California*

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Various pathways have been postulated for nicotine degradation involving an initial attack at either the pyridine or the pyrrolidine rings. The evidence for the suggested pathways has been provided mainly by the isolation of a variety of compounds from bacterial growth media (1, 2), tobacco seed infusions (3), fermented tobacco leaves (4), and the urine of animals that previously had been fed nicotine (5, 6). Unfortunately, because of the complexity of these systems it is not certain whether the isolated products are directly or indirectly derived from nicotine, nor is the sequence of their appearance clearly established. In an effort to avoid the difficulties inherent in the use of complex systems, studies of nicotine metabolism were attempted at the enzyme level employing crude and fractionated extracts derived from a bacterium.

### EXPERIMENTAL

The organism (designated as strain P-34) employed is a gram-negative rod isolated from soil by enrichment culture techniques. It is capable of using nicotine as its sole source of carbon and energy but its growth is stimulated by yeast extract. It was grown in a medium having the following composition in grams per 100 ml.: 1.33  $K_2HPO_4 \cdot 3H_2O$ , 0.4  $KH_2PO_4$ , 0.1  $(NH_4)_2SO_4$ , 0.1 yeast extract, 0.4 nicotine, and the following trace salts; 0.01  $MgSO_4 \cdot 7H_2O$ , 0.002  $CaCl_2 \cdot 2H_2O$ , 0.004  $MnSO_4 \cdot 4H_2O$ , 0.0002  $FeSO_4 \cdot 7H_2O$ . The trace salts were dissolved in 0.1 N HCl at 100 times the final medium concentration, autoclaved, and added aseptically to the medium in the required amounts.

For the preparation of large batches of cells, growth was carried out in 12-liter round bottom flasks containing 7 liters of medium. The inoculated medium was aerated with sterile air and incubated at room temperature. After the culture reached the maximum stationary phase (approximately 60 hours), the cells were harvested in a Sharples centrifuge. The unwashed cell paste was stored at  $-18^\circ$  until needed. Yields were of the order of 6.5 gm. wet weight of cells per liter of medium.

Cell-free extracts were prepared according to the method of McIlwain (7) by grinding a mixture of 10 gm. of partially thawed cells and 25 gm. of levigated alumina in a cold mortar. After grinding for 10 to 14 minutes at room temperature, the cell-alumina mixture became "tacky." The resulting paste was extracted with 30 ml. of 0.01 M potassium phosphate buffer, pH 7, for 15 minutes at room temperature and for 45 minutes at  $-18^\circ$ .

\* This work was supported by a grant from the Tobacco Industry Research Committee. Preliminary results were presented to the Society of American Bacteriologists, May 1957.

† Present address, Department of Medical Microbiology, University of Southern California School of Medicine.

4°. The alumina, unbroken cells, and cell debris were removed by centrifugation at  $18000 \times g$  for 60 minutes at  $4^\circ$ . The resulting clear yellow supernatant fluid constituted the crude extract and contained, on the average, 22 mg. of protein per ml.

Ammonium sulfate fractionation of the crude extract was carried out at room temperature by the addition of the required amount of solid ammonium sulfate (8). After the addition of the ammonium sulfate, the precipitated protein was removed by centrifugation at  $4^\circ$  for 10 minutes at  $18000 \times g$ . The supernatant solution was decanted, the pellet drained by inversion and dissolved in 0.01 M potassium phosphate buffer, pH 7, to give a final volume of approximately one-fourth that of the starting crude extract. All enzymatic fractions were stored at  $-18^\circ$  if not used immediately.

Protein was determined by trichloroacetic acid precipitation (9), crystalline egg albumin being used as the standard. The optical density at 540 m $\mu$  was determined in a Bausch and Lomb Spectronic-20.

Ultraviolet absorption spectra were determined with a Beckman model DU spectrophotometer. Reaction mixtures were prepared for spectrophotometry by adding them to 2 volumes of 0.1 N HCl, removing the precipitated protein by centrifugation, and, if required, diluting the clear supernatant solution with additional 0.1 N HCl.

Oxygen consumption and carbon dioxide production were determined by conventional manometric techniques (10).

Reaction mixtures were chromatographed on Whatman No. 1 paper by the ascending technique. The solvent was an 85:5:30 mixture of *n*-butanol, benzene, and 0.2 M sodium acetate buffer, pH 5.6 (11). The alkaloids were located by exposing the dried chromatograms to cyanogen bromide vapors for 1 hour followed by spraying with  $\beta$ -naphthylamine (a Koenig's reaction), by ultraviolet absorption under a Mineralite lamp, or by treating the paper with Dragendorff's reagent.<sup>1</sup>

### RESULTS

*Oxidation of Nicotine by Crude Extracts*—Crude extracts prepared from several independently grown batches of cells usually oxidized nicotine at a slow but definite rate. The amount of oxygen consumed varied from extract to extract and in many cases no oxidation was observed. Furthermore, fresh extracts which oxidized nicotine lost this ability upon storage at  $-18^\circ$ .

<sup>1</sup> Prepared by mixing 5 parts of solution A (0.85 gm. of bismuth subnitrate, 40 ml. of distilled water, and 10 ml. of glacial acetic acid) with 5 parts of solution B (8 gm. of potassium iodide in 20 ml. of distilled water), and adding 20 ml. of glacial acetic acid and 100 ml. of distilled water.

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TABLE I

## Oxidation of nicotine by methylene blue-supplemented crude extracts

Experimental conditions: 1  $\mu$ mole nicotine, 102  $\mu$ moles potassium phosphate buffer, pH 7, 1  $\mu$ mole methylene blue where noted, 1 ml. crude extract; total volume 2.0 ml., gas phase air, 30°. The age of the extract represents days of storage, after preparation, at  $-18^\circ$ .

Experiment No.	Age of extract days	Methylene blue	Oxygen consumed after 60 min. $\mu$ l.
1	0	-	48
	0	+	78
	4	-	2
2	0	+	65
	0	+	71
	1	+	67

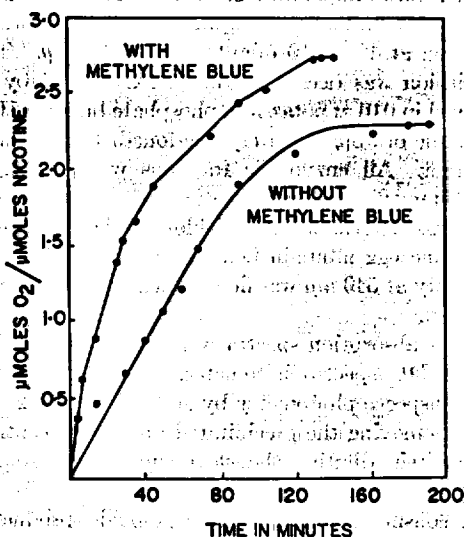


Fig. 1. The oxidation of nicotine by a crude extract. In the presence of methylene blue, the reaction mixture contained 5  $\mu$ moles of nicotine, 120  $\mu$ moles of potassium phosphate buffer, pH 7, and 1 ml. of crude extract. In the absence of methylene blue, the reaction mixture contained 1  $\mu$ mole of nicotine, 88  $\mu$ moles of potassium phosphate buffer, pH 7, and 1 ml. of crude extract. Data corrected for oxygen consumption in the absence of substrate. Total volume, 2.0 ml., gas phase air, 30°.

TABLE II

## Oxygen consumption and carbon dioxide formation during nicotine oxidation by methylene blue-supplemented crude extracts

Experimental conditions: 5  $\mu$ moles nicotine, 120  $\mu$ moles potassium phosphate buffer, pH 7, 1.25  $\mu$ moles methylene blue, 11 mg. (Experiment 1) and 17.3 mg. (Experiment 2) crude extract; total volume 2.0 ml., gas phase air, 30°.

Experiment No.	$\mu$ Moles oxygen consumed		$\mu$ Moles carbon dioxide produced	
	Total	Per $\mu$ mole of nicotine	Total	Per $\mu$ mole of nicotine
1	14.5	2.9	1.94	0.39
2	13.8	2.8	1.88	0.38

Oxidative activity in aged extracts was restored by the addition of methylene blue, brilliant cresyl blue, and 2,6-dichlorophenolindophenol, but not by a number of cofactors, by metallic ions,<sup>3</sup> or by cell debris.

In addition to restoring oxidative activity lost during storage, methylene blue rendered active those extracts which did not oxidize nicotine when freshly prepared, and stimulated the rate of oxygen consumption in those extracts which initially had oxidative activity (Table I). No oxidation of nicotine was observed in the absence of enzyme thus ruling out a photochemical oxidation of nicotine by methylene blue (12) under the conditions employed.

Methylene blue also affected the manner in which nicotine oxidation occurred. Whereas unsupplemented extracts oxidized nicotine at a constant rate, methylene blue-supplemented crude extracts oxidized nicotine in a series of steps of decreasing oxidative rate (Fig. 1). These rate changes occurred after the consumption of nearly 0.5  $\mu$ mole of oxygen, or some integral multiple of 0.5, per  $\mu$ mole of nicotine until a total of approximately 3  $\mu$ moles of oxygen per  $\mu$ mole of nicotine was consumed. The data presented indicate rate changes after the consumption of 0.6, 1.6, 1.9, 2.5, and 2.8  $\mu$ moles of oxygen per  $\mu$ mole of nicotine. The position of the changes differed from extract to extract, and, in most experiments, the first change in rate occurred after the consumption of 1  $\mu$ mole of oxygen per  $\mu$ mole of nicotine. Total oxygen consumption was usually 3  $\mu$ moles per  $\mu$ mole of nicotine, but occasionally somewhat smaller or larger values were observed. Assuming that 2 electrons were transferred per oxidative step, the typical crude extract catalyzed six oxidative reactions in nicotine degradation. No significant amounts of carbon dioxide were released up to this point of oxidation (Table II), suggesting that the fundamental carbon skeleton of nicotine might still be intact.

**Chromatography of Reaction Mixtures**—Chromatograms run on reaction mixtures stopped at the first rate change (after the consumption of approximately 0.5  $\mu$ mole of oxygen per  $\mu$ mole of nicotine) showed no residual nicotine, but did show another compound with a lower  $R_F$ . After the consumption of approximately 1  $\mu$ mole of oxygen per  $\mu$ mole of nicotine, neither nicotine nor the previous compound was detected, and a single new ultraviolet light-absorbing and Dragendorff reagent-positive spot was observed (Table III).<sup>3</sup> It would appear not only that products had accumulated at the points at which change of rate occurred, but also that essentially all of the initial substrate was consumed before oxidation of the subsequent substrate was initiated.

**Changes in Ultraviolet Absorption Spectra**—With the consumption of 0.5  $\mu$ mole of oxygen per  $\mu$ mole of nicotine, a marked change in the ultraviolet absorption spectrum of the reaction mixture was observed. The absorption maximum at 260  $m\mu$  due to the pyridine moiety of nicotine disappeared, and in its place 2 absorption maxima appeared, a rather sharp and intense peak at 232  $m\mu$ , and a peak of lower extinction at 295  $m\mu$ . These peaks were absent after the consumption of 1  $\mu$ mole of

<sup>3</sup> The following cofactors and metallic ions were tried individually and in combination with negative results: ATP, ADP, DPN, TPN, CoA, GSH, riboflavin, riboflavin-5-phosphate, cytochrome C,  $MgSO_4 \cdot 7H_2O$ ,  $MnSO_4 \cdot 4H_2O$ ,  $CuSO_4 \cdot 5H_2O$ ,  $FeSO_4 \cdot 7H_2O$ ,  $FeCl_3$ ,  $ZnSO_4 \cdot 6H_2O$ ,  $CoCl_2 \cdot 6H_2O$ , and  $MoO_3$ .

<sup>4</sup> Although the observed  $R_F$  values were very close, the compound with an  $R_F$  of 0.12 gave a much redder color with Dragendorff's reagent than the compound with an  $R_F$  of 0.09.

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TABLE III

Paper chromatography of reaction mixtures after consumption of 0.5 and 1  $\mu$ mole of oxygen per  $\mu$ mole of nicotine

Oxygen consumed $\mu$ moles/ $\mu$ mole nicotine	<i>R<sub>F</sub></i>		
	CNBr	Ultraviolet absorption	Dragendorff's reagent
0	0.33	*	*
0.57	—†	0.12	0.12
1.05	—†	0.09	0.09
Nicotine control	0.32	0.32	0.32

\* Not employed.

† No spot detected.

oxygen per  $\mu$ mole of nicotine. However, a new absorption maximum at 290  $m\mu$  was observed (Fig. 2). These results supported the chromatographic evidence for the temporary accumulation of intermediates, and showed that the first two of these substances could be easily distinguished from one another and from nicotine by means of their absorption in the ultraviolet.

**Oxidation of Nicotine by Ammonium Sulfate Fractions**—By fractionating the crude extract with ammonium sulfate in increments of 20 per cent of saturation with respect to ammonium sulfate, two preparations were obtained which oxidized nicotine when supplemented with methylene blue. Although fractionation did not lead to a large increase in specific activity, it did recover a major portion of the initial crude activity (Table IV). What is more important, fractionation with ammonium sulfate separated the material responsible for the initial oxidative step from the subsequent ones (Fig. 3).

The 20 to 40 fraction, when dissolved in buffer, gave an intensely yellow solution. It oxidized nicotine in the presence of methylene blue, and oxidation ceased after the consumption of 0.5  $\mu$ mole of oxygen per  $\mu$ mole of nicotine (Fig. 3).

When nicotine and methylene blue were present in excess, the rate of nicotine oxidation was limited by the concentration of enzyme protein over the experimentally determined range of from 0.11 mg. to 1.1 mg. per ml. In the presence of an excess of nicotine and of enzyme, methylene blue limited the rate of oxidation up to a concentration of  $2.5 \times 10^{-4}$  M, in reasonably close agreement with that observed with crude extracts. In the presence of an excess of enzyme and of methylene blue, nicotine limited the rate of oxidation up to a concentration of approximately  $5 \times 10^{-3}$  M (Fig. 4).

After the oxidation of nicotine by the 20 to 40 fraction ceased, the ultraviolet absorption spectrum and the chromatographic behavior of the reaction mixture were identical to that observed at the 0.5  $\mu$ mole oxygen rate change when nicotine was oxidized by a crude extract.

The 40 to 60 fraction was obtained as a light green pellet. Relatively concentrated solutions of this fraction were deep green in color when dissolved in 0.01 M potassium phosphate buffer, pH 7. This fraction oxidized nicotine only in the presence of methylene blue, with the consumption of 1  $\mu$ mole of oxygen per  $\mu$ mole of nicotine (Fig. 3). At the end of the oxidation, the reaction mixture had the ultraviolet absorption spectrum and the chromatographic properties present at the 1  $\mu$ mole oxygen rate change obtained with the crude extract. The 40

to 60 fraction apparently contained the enzymes responsible for both the first and second oxidative steps in nicotine degradation. The properties of this fraction will be more fully described in a subsequent paper.

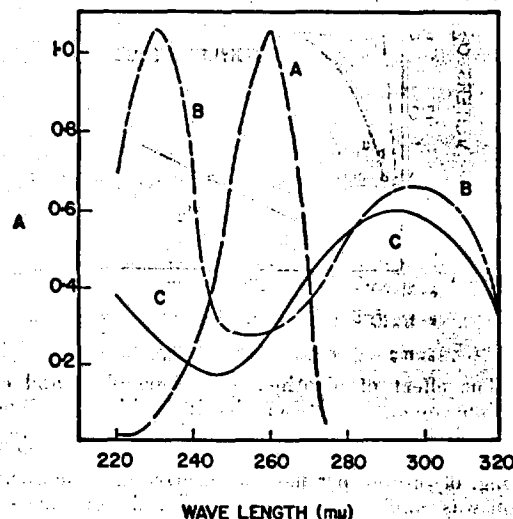


FIG. 2. The ultraviolet absorption spectra of reaction mixtures. Curve A, zero time control. Curve B, after the consumption of 0.53  $\mu$ mole of oxygen per  $\mu$ mole of nicotine. Curve C, after the consumption of 1.04  $\mu$ moles of oxygen per  $\mu$ mole of nicotine.

TABLE IV

Fractionation of crude extracts with ammonium sulfate

Ammonium sulfate fraction	Total protein	Total units*	Specific activity	Recovery of units
% saturation	mg.		units/mg.	%
Crude	543	4724	9	
0-20	7	0	0	0
20-40	120	2712	23	57
40-60	222	1376	6	29
60-80	3	0	0	0

\* A unit of enzyme activity is defined as 1  $\mu$ l. oxygen consumed per 10 minutes during the maximum rate of oxygen consumption

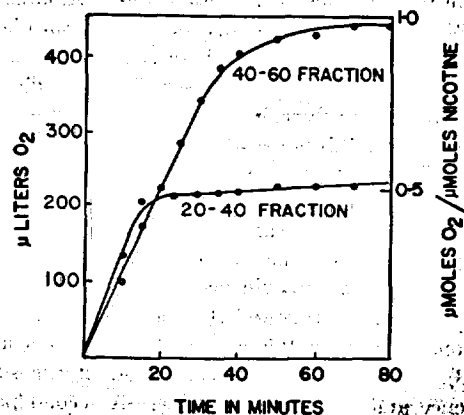


FIG. 3. The oxidation of nicotine by ammonium sulfate fractionated crude extracts. The reaction mixture contained 20  $\mu$ moles of nicotine, 58  $\mu$ moles of potassium phosphate buffer, pH 7, 1.25  $\mu$ moles of methylene blue, and the following ammonium sulfate fractions: 7.5 mg. 20 to 40 fraction and 18.5 mg. 40 to 60 fraction. No oxygen consumption was observed in the absence of nicotine, methylene blue, or the enzyme fractions.

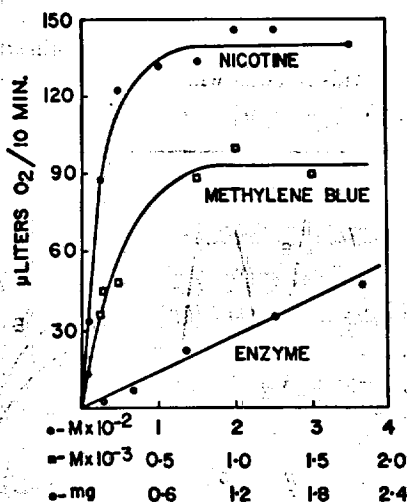
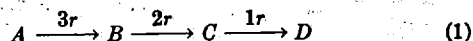


FIG. 4. The effect of nicotine, methylene blue, and enzyme concentrations upon the rate of nicotine oxidation. When not employed as the variable, the concentrations of nicotine, methylene blue, and the 20 to 40 fraction were  $2 \times 10^{-2}$  M,  $1 \times 10^{-3}$  M, and 28.4 mg. of protein per flask, respectively. When the 20 to 40 fraction was employed as the variable, the enzyme solution contained 2.2 mg. of protein per ml.

#### DISCUSSION

The observed changes in rate during oxidation of nicotine by crude extracts in the presence of methylene blue are puzzling. One could devise a model system exhibiting similar changes in rate in which a series of single step oxidations proceed simultaneously but at successively decreasing rates. In such a system, the rate of oxygen uptake would change with the disappearance of each member of the sequence in turn, and at the change, all succeeding members would be present in proportion to the difference in their rates of formation. Thus if



at the indicated rates,  $r$ , the initial rate of oxygen uptake would be  $6r$  and would change to  $3r$  with the exhaustion of  $A$ . At this point,  $B$ ,  $C$ , and  $D$  would be present in a ratio of 1:1:1. Although the rate changes during nicotine oxidation correspond to points of temporary accumulation of intermediates, the situation is quite different from the hypothetical example in that the data show that none of the serially accumulated products are metabolized until their successive precursors have been exhausted. That is,  $B$  is not oxidized until  $A$  is exhausted.

The mechanism responsible for this unique situation is not known. The possibility that each precursor competes with its product for an enzyme site and thus inhibits product oxidation was ruled out, at least in the case of the 0.5  $\mu$ mole of oxygen product, by the following evidence. The 40 to 60 fraction, which oxidizes both nicotine and the product that accumulates after the consumption of 0.5  $\mu$ mole of oxygen per  $\mu$ mole of nicotine (13), oxidized the latter compound at the same rate in the presence and absence of nicotine. A second possibility is that accumulation and changes of rate are a result of some cofactor deficiency, a cofactor shared in common by several of the enzymes involved in nicotine oxidation. This mechanism would demand that the cofactor exist as a ternary complex between enzyme, substrate, and cofactor, and that the enzyme-substrate complex have a very high affinity for the cofactor

relative to the affinity of the succeeding enzyme-substrate complex.

The changes in the absorption spectrum during nicotine oxidation provide a clue as to the nature of the initial oxidative step. Nicotine, by virtue of its pyridine moiety, absorbs strongly at 260  $m\mu$ . It is known (14) that the introduction of a double bond in conjugation with the pyridine ring results in the appearance of a new absorption maximum at 234  $m\mu$  accompanied by a bathochromic shift of the 260  $m\mu$  absorption maximum. The addition of a second double bond in conjugation with the pyridine ring results in an additional bathochromic shift accompanied by a complete loss of the characteristic absorption maximum associated with the pyridine nucleus (14). One possible interpretation of our spectrophotometric data is that the initial reaction during the oxidation of nicotine produces an unsaturation of the pyrrolidine ring. However, the failure to observe a Koenig's reaction subsequent to the consumption of 0.5  $\mu$ mole of oxygen per  $\mu$ mole of nicotine (Table III) implicates either the nitrogen or the  $\alpha$ -carbons of the pyridine ring as the sites of attack (15). The two possible reactions in this case would be the addition of oxygen to the pyridine nitrogen to yield a pyridine- $N$ -oxide derivative of nicotine, or the addition of oxygen to either  $\alpha$ -carbon to yield a pyridone derivative of nicotine. The possibility that the product was an  $N$ -oxide seemed unlikely when it was found that pyridine- $N$ -oxide itself, in 0.1 N HCl, has but a single absorption maximum located at 255  $m\mu$  (13). A pyridine- $N$ -oxide derivative of nicotine would be expected to possess an analogous absorption spectrum in the ultraviolet. On the other hand, an authentic sample of 2-pyridone, in 0.1 N HCl, exhibited 2 absorption maxima, one at 227 and the other at 297; the latter absorption maximum had a lower extinction (13). Thus it seems probable that the first oxidative product is a pyridone and that the organism P-34 initiates the oxidation of nicotine in a manner similar to the pyridine pathway postulated by Frankenburg and Vaitekunas (3). In the following paper, the isolation and identification of this compound will be described.

#### SUMMARY

Crude cell-free extracts prepared from a soil bacterium capable of growing at the expense of nicotine as the sole source of carbon and nitrogen degraded nicotine with the consumption of 3  $\mu$ moles of oxygen per  $\mu$ mole of nicotine when supplemented with methylene blue. No carbon dioxide was formed up to this level of oxidation.

With crude extracts, nicotine oxidation proceeded through a series of sharp changes of rate occurring after the uptake of 0.5, or multiples of 0.5,  $\mu$ mole of oxygen per  $\mu$ mole of nicotine. Chromatographic evidence and ultraviolet absorption data indicated that each point of change of rate coincided with the temporary accumulation of intermediates in the oxidation sequence, and that none of the serially accumulated intermediates are oxidized until their precursors are exhausted. After the consumption of 0.5  $\mu$ mole of oxygen per  $\mu$ mole of nicotine, with the use of either a crude extract or an ammonium sulfate fractionated enzyme, the absorption maximum at 260  $m\mu$  resulting from nicotine disappeared, and a compound having absorption peaks at 232 and 295  $m\mu$  appeared. With the additional uptake of 0.5  $\mu$ mole of oxygen per  $\mu$ mole of nicotine, these peaks disappeared and a new absorption maximum at 290  $m\mu$  was observed.

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The nature of the absorption spectrum of the first oxidative product and its failure to give a Koenig's reaction suggests a primary attack at the pyridine moiety of nicotine to yield a pyridone substituted at an  $\alpha$ -carbon of the pyridine ring.

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TIRC Grant #209  
(cf. #86)**CONFIDENTIAL**Report No. 6  
October 1958 - May 1959Sydney C. Rittenberg, Ph.D.  
University of Southern California

May 19, 1959

The bacterial degradation of nicotine and related compounds.

(Not included: certain additional information obtained on the second product. A copy of a paper on this will be received soon. It is being sent to the Journal of Biological Chemistry.)

The third oxidative product:

Since our last report in October 1958 in which very cursory information on the third oxidative step in the bacterial oxidation of nicotine was given, we have pursued this phase of the problem further. The initial investigations along this line were directed towards the further elucidation of the conditions necessary for formation of the third oxidative product. These investigations showed the following conditions optimal for the conversion of nicotine, 6-hydroxynicotine, or 6-hydroxypseudonornicotine to third product with the uptake of 1.5, 1.0 and 0.5  $\mu\text{m}$  of oxygen per  $\mu\text{m}$  of substrate respectively.

- a) The fraction of the crude enzyme precipitating between 40 and 60 per cent ammonium sulfate saturation contains the enzyme system necessary for this conversion.
- b) The pH optimum for formation of third product from second product lies between 7.5 and 8.0.
- c) The product is formed in the presence of methylene blue or brilliant cresyl blue and the 40-60 ammonium sulfate fraction. In the case of the former dye the oxidation proceeds slowly past the third product stage and in the case of the latter stops at the third step.
- d) The optimum concentration of BCB was found to be 0.08  $\mu\text{m}$  of the dye under conditions where enzyme concentration was not limiting.

With the conditions for the formation of the third product established, we next attempted a large scale isolation of the compound. This was done in the Warburg apparatus using 800 mg of 6-hydroxynicotine as the starting material. The oxidation was followed in a Warburg vessel containing an aliquot of the main reaction mixture which was shaken in the Warburg in an Erlenmeyer flask. The progress of the formation of the product was also followed spectrophotometrically using diluted aliquots from the main flask. The decrease in the absorbance at 290 m $\mu$  (6HN) and the increase at 360 m $\mu$  (third product) were measured. When oxidation ceased in the Warburg vessel and the 360 absorbance remained stationary, the reaction was presumed to be complete.

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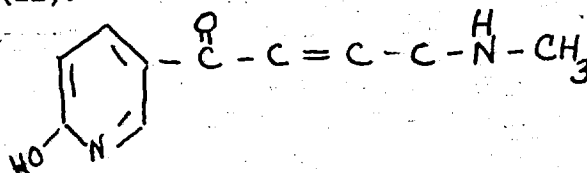
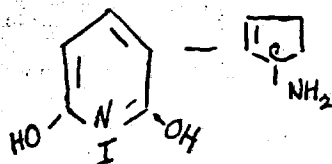
The reaction mixture was deproteinized by heating at 65° C for 15 minutes and the precipitated protein removed by centrifugation. The clear supernate was brought to pH 1.0 with concentrated HCl and put on a Dow 50 ion exchange column in the hydrogen form. After washing with water and 0.3 M NH<sub>4</sub>OH the compound was eluted with 0.9 M NH<sub>4</sub>OH. All of the 20 ml fractions showing appreciable 360 mμ absorbance were combined and taken to dryness in vacuo.

The yellow-brown residue was extracted with amyl alcohol from which, upon concentration and slow cooling, needles separated out. After five recrystallizations from butanol 200 mg. of faintly pink colored needle-shaped crystals were obtained. The isolated compound has the following physical and chemical properties:

- It forms a yellowish solution in water, alcohol, acetic acid and mineral acids. It is relatively insoluble in ether.
- It adsorbs strongly in the UV having 2 maxima at 290 and 360 mμ. The 360/290 ratio of the compound is 5.15.
- The uncorrected melting point on a block is 258-260 C.
- The compound is homogeneous when chromatographed on paper and gives a violet spot when sprayed with acid FeCl<sub>3</sub>. The compound gives a blue color characteristic of dihydroxy pyridines when tested with the Folin-Ciocalteu phenol reagent.
- The elemental analysis calculated for C<sub>10</sub>H<sub>12</sub>N<sub>2</sub>O<sub>2</sub> was:

	Calc.	Found
C	62.5	C 61.22
H	6.25	H 6.31
N	14.55	N 14.22

From the above data two possible basic structures may be postulated for third product, a dihydroxy-N-methyl myosmine (I) or a compound containing a double bond in the open side chain (II).



At present the evidence is more in favor of I (the second hydroxyl may be in the 2,4 or 5 position) because of the typical diphenol type reactions obtained with ferric chloride and the Folin reagent. The compound, whatever its structure, is apparently new to nicotine chemistry since nothing resembling its properties has been found in the literature.

A strange situation exists in the case of the isolated compound however. Under none of the conditions so far employed have we been able to obtain further oxidation of the purified compound. These conditions include tests of enzymes, crude and fractionated, resting cells, and dried cells, all with and without methylene blue present.

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We have several explanations for this anomalous behavior. First the product may be altered during the isolation procedure and second this product may not be on the main oxidative pathway. With respect to the second alternative it is possible that the BCB is acting as a poisoning agent that forces the formation of this product due to an alteration in the oxidation-reduction potential of the system. In favor of the second hypothesis is the finding that the material formed in the reaction mixture before chemical manipulations and the purified material are identical chromatographically and spectrophotometrically.

There is a third possibility which we have not as yet investigated. This involves the formation of the isolated product from some precursor which is not itself on the main pathway. Since the second product can exist in three forms dependent on pH it could well be that one of these forms gives rise to the isolated product and the other gives rise to the true third oxidation product.

We have carried out a few very preliminary studies on the formation of the fourth product and as yet are unable to state anything definite about its nature except to say that all UV absorbance disappears at this stage indicating that the pyridine ring has been ruptured. The fourth product has been found on paper chromatograms of reaction mixtures using a diazotizing reagent as the indicator.

In the next six months we hope to more fully characterize the isolated third product and to complete the identification of the substance. We expect to obtain a more concise picture of the biological significance of this compound and to elucidate its position in the oxidation of nicotine by this organism.

We will investigate the conditions necessary for the formation of the fourth product and will attempt to isolate and identify the compound in the near future.

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TRC Grant  
#209

## The Bacterial Oxidation of Nicotine

### III. THE ISOLATION AND IDENTIFICATION OF 6-HYDROXYPSEUDOOXYNICOTINE\*

LAWRENCE I. HOCHSTEIN† AND SYDNEY C. RITTENBERG

*From the Department of Bacteriology, University of Southern California, Los Angeles, California*

(Received for publication, September 2, 1959)

Previous studies in this series (1, 2) have established that an enzyme fraction, obtained from the nicotine-oxidizing bacterium P-34, catalyses the oxidation of nicotine and 6-hydroxynicotine with the consumption of 1  $\mu$ mole and 0.5  $\mu$ mole of oxygen, respectively, per  $\mu$ mole of substrate. This paper reports on the isolation of the product of nicotine and 6-hydroxynicotine oxidation by the above mentioned enzyme fraction and the identification of the isolated compound as 6-hydroxypseudooxynicotine.

#### EXPERIMENTAL

The preparation of cell-free extracts, ultraviolet spectrophotometry, paper chromatography, optical rotation, and melting point determinations as well as manometric determinations of oxygen consumption and carbon dioxide release were performed as previously described (1, 2). Ammonia was collected by the Conway microdiffusion technique (3), and determined by direct nesslerization of the trapped ammonia.

6-OHN<sup>1</sup> was isolated from growth medium as the silicotungstic acid salt and purified by a modification of the previously described method (2). A solution of the free base, regenerated from its silicotungstic acid salt (4), was acidified with hydrochloric acid to pH 2, and placed on a Dowex 50 column in the hydrogen form (1.5  $\times$  21 cm). The column was washed with water until the effluent exhibited a low and constant absorbancy at 232 and 295  $m\mu$ . A gradient elution schedule was then initiated employing 3 M ammonium hydroxide in the reservoir and 2.5 liters of distilled water in the mixing chamber. Fractions were collected in 18-ml lots; after 216 ml of effluent had been collected, 6-OHN, as judged by the increase in absorbancy at 232 and 295  $m\mu$ , appeared in the following 108 ml. The fractions containing 6-OHN were pooled, acidified to pH 2 with hydrochloric acid, and evaporated to dryness. 6-OHN was extracted from the dry residues with boiling Skellysolve B and crystallized from the same solvent.

#### RESULTS

**Oxidation of 6-OHN**—The 40-60 fraction,<sup>2</sup> which catalyzed the aerobic oxidation of nicotine only in the presence of methylene blue (1), oxidized 6-OHN in the absence of the dye. Oxidation

ceased after the consumption of 0.5  $\mu$ mole of oxygen per  $\mu$ mole of 6-OHN. No production of carbon dioxide or of significant quantities of ammonia was observed (Table I).

The rate of oxidation exhibited a marked dependence upon pH. When determined in Tris-maleate buffer, the optimum occurred at pH 8 (Fig. 1). 6-OHN oxidation was a function of the enzyme concentration up to 1.05 mg protein per ml of reaction mixture (Fig. 2). When the enzyme concentration was at excess (3.7 mg protein per ml of reaction mixture), the rate of 6-OHN oxidation was independent of the substrate concentration over the range tested ( $7 \times 10^{-4}$  M to  $1.05 \times 10^{-2}$  M).

At the termination of 6-OHN oxidation, reaction mixtures contained a substance having an absorption maximum at 289  $m\mu$  when determined in 0.1 N hydrochloric acid. When the spectra were determined in 0.1 N sodium hydroxide, the maximum exhibited a reversible bathochromic shift to 310  $m\mu$ . Identical absorption characteristics were exhibited by reaction mixtures obtained by incubating nicotine with crude enzyme and methylene blue and stopping the oxidation when 1  $\mu$ mole of oxygen per  $\mu$ mole of nicotine had been consumed. Three volumes of 0.1 N HCl were added to samples of both types of reaction mixtures and the precipitated protein was removed by centrifugation. Portions of the supernatant were spotted on Whatman No. 1 paper and chromatographed with butanol-benzene-0.2 M sodium acetate buffer, pH 5.6 (85:5:30) as the solvent and Dragendorff's reagent as the indicator. Both types of reaction mixtures contained a compound of  $R_f$  0.09, which was not evident in mixtures devoid of substrate. In the solvent system used, nicotine and 6-OHN had  $R_f$  values of 0.32 and 0.15, respectively. These data indicate that the compounds formed by the crude extract, at the expense of nicotine, and by the 40-60 fraction, at the expense of 6-OHN, were identical.

**Enzymatic Synthesis and Isolation of Product**—In a typical experiment, the following components were added to each of two 250-ml Erlenmeyer flasks: 1250  $\mu$ moles of 6-OHN, previously adjusted to pH 7.9 with hydrochloric acid; 2 ml of a 40-60 fraction (116 mg of protein); and water to a total volume of 20 ml. The flasks were shaken in a 30° water bath. The reaction was followed manometrically by simultaneously incubating 2 ml of the above reaction mixture in a Warburg flask. When oxidation ceased, the reaction mixtures were pooled, brought to 70° for 5 minutes, and the denatured protein was removed by centrifugation.<sup>3</sup> The clear supernatant was lyophilized and the residue

\* This work was supported by a grant from the Tobacco Industry Research Committee.

† Present address, Biochemical Research Laboratory, Elgin State Hospital, Elgin, Illinois.

<sup>1</sup> The abbreviations used are: 6-HPO, 6-hydroxypseudooxynicotine; 6-OHN, 6-hydroxynicotine.

<sup>2</sup> That portion of the crude extract which precipitated at 40 to 60 per cent saturation with respect to ammonium sulfate is designated as the 40-60 fraction.

<sup>3</sup> Failure to immediately denature the enzyme at this stage results in the formation of a blue pigment. This pigment formation in nicotine degradation will be discussed in a subsequent publication.



TABLE I

## Oxidation of 6-OHN by 40-60 Enzyme Fraction

The complete system contained the following in a total volume of 2.0 ml: 14  $\mu$ moles of 6-OHN; 1.25  $\mu$ moles of methylene blue (MB); 10  $\mu$ moles of potassium phosphate buffer, pH 7; 2.1 mg of 40-60 fraction. Gas phase air; 30°. The 40-60 fraction was dialyzed overnight against distilled water.

Condition	$\mu$ Moles per $\mu$ mole of 6-OHN		
	Oxygen uptake	CO <sub>2</sub> formed	NH <sub>3</sub> formed
Complete system	0.52	0	
Minus 6-OHN	0	0	
Minus MB	0.46	0	0.20
Minus MB and 6-OHN	0	0	0.18

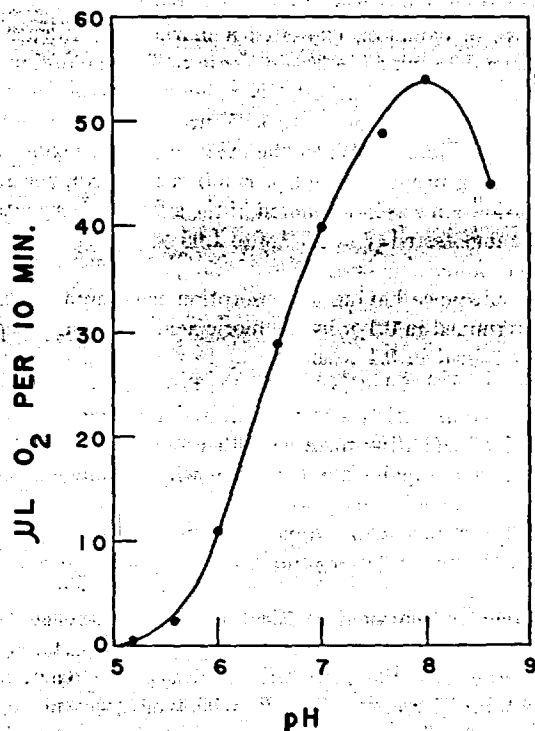


FIG. 1. The effect of pH on the oxidation of 6-OHN. The reaction mixtures contained the following in a total volume of 2.0 ml: 6-OHN, 14  $\mu$ moles; Tris-maleate buffer, 100  $\mu$ moles, at the desired pH; 40-60 fraction, 1.4 mg protein. Gas phase air, 30°.

extracted with 50 ml of absolute ethanol previously acidified with dry hydrochloric acid. The alcoholic extract was warmed and anhydrous ethyl ether was slowly added with constant shaking until a permanent faint turbidity was obtained. The solution then was cooled to room temperature and placed at -10° for at least 12 hours. The resulting precipitate was filtered, washed with cold (-10°) absolute ethanol, and dried in a vacuum. The washings and the filtrate were combined and the ether precipitation repeated through several cycles until further treatment failed to yield a precipitate (total yield of material was 695 mg). The initial precipitates in a series were usually somewhat resinous and dark colored. Succeeding fractions became lighter in color and amorphous, or even crystalline, if the ether additions were carefully controlled, and, in 0.1 HCl, they showed a progressive increase in absorption at 289 m $\mu$ . A small portion, 75 mg, of

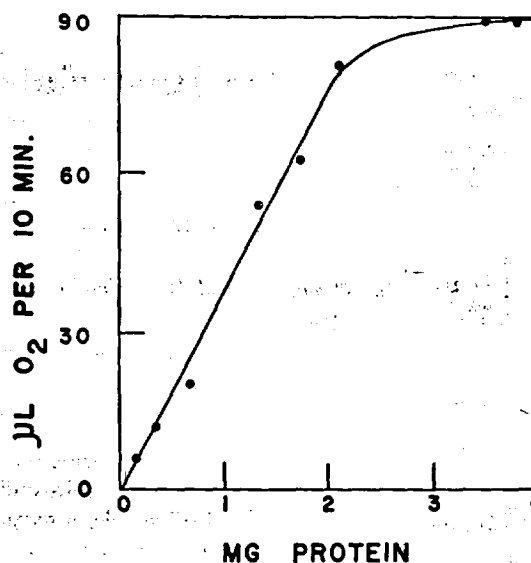


FIG. 2. The effect of enzyme concentration on the oxidation of 6-OHN. The reaction mixtures contained the following in a total volume of 2.0 ml: 6-OHN, 14  $\mu$ moles; Tris-maleate buffer, pH 8.0, 100  $\mu$ moles; and 40-60 fraction protein as indicated. Gas phase air, 30°.

a nonresinous fraction of high absorption was redissolved in acid ethanol, decolorized with Norit A, and reprecipitated with ethyl ether as above. The final product, 46 mg, consisted of white crystalline needles that melted at 157-158° (uncorrected).

The isolated product had a single absorption maximum whose position was pH dependent (Fig. 3). At a pH of less than 8 the peak occurred at 289 m $\mu$  ( $\epsilon$  = 16,000), at a pH between 8 and 12 at 328 m $\mu$  ( $\epsilon$  = 22,000), and at a pH greater than 12 at 310 m $\mu$  ( $\epsilon$  = 21,000).

The absorption spectrum of the isolated product suggested the presence of a new double bond in conjugation with the pyridine ring (5). This, coupled with the stoichiometry of oxygen consumption and the failure to detect optical activity in the product further indicated that oxidation resulted in the destruction of the molecule's center of asymmetry located at carbon 5 of the pyrrolidine ring. Dehydrogenation at this position would yield 6-hydroxy-N-methylmyosmine; however elemental analysis<sup>4</sup> indicated that an hydrolytic as well as an oxidative step had occurred and suggested that the product isolated was a dihydrochloride of 6-HPO:



Calculated: C 44.94, H 5.99, N 10.49, Cl 26.59

Found: C 45.44, H 5.95, N 10.55, Cl 27.27

The identity of the isolated product was established as 6-HPO by comparing it to the product of the enzymatic hydroxylation of pseudoxyntine.<sup>5</sup> The enzyme fraction employed has been shown to hydroxylate a number of compounds related to nicotine, including pseudoxyntine, at the 6-position of the pyridine ring (6). It was found that following hydroxylation of pseudoxyntine, the original absorption maxima located at 223 m $\mu$  and 264 m $\mu$  were replaced by a single maximum

<sup>4</sup> The analysis was performed by Dr. A. Elek, Elek Micro Analytical Laboratories, Los Angeles, California.

<sup>5</sup> Kindly supplied by Dr. C. H. Rayburn.

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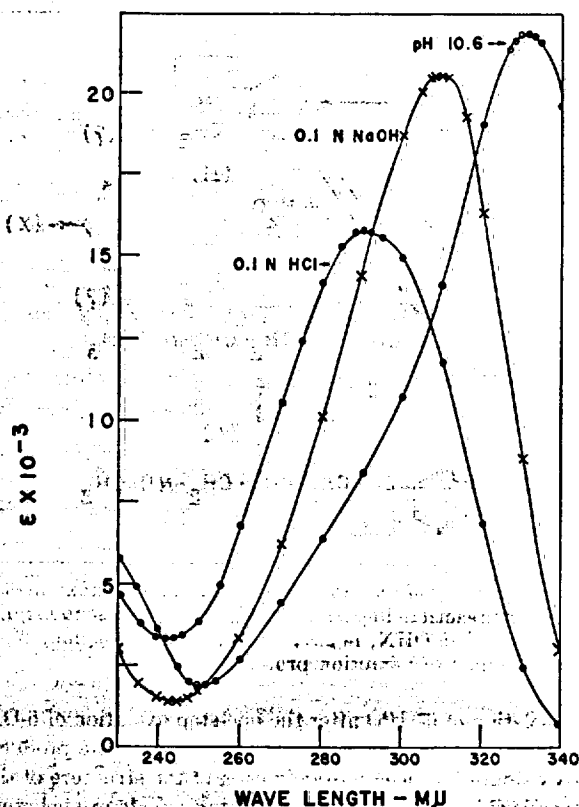


Fig. 3. The absorption spectrum of the isolated product. 0.1 N HCl, ●—●; pH 10.6, ○—○; 0.1 N NaOH, ×—×.

whose location depended upon pH in a manner identical to the behavior of the product of 6-OHN oxidation. In addition, the chromatographic characteristics of the products from both substrates were identical (Table II).

**Metabolism of 6-HPO**—Nicotine-grown resting cells oxidized 6-HPO at approximately the same rate as they oxidized nicotine. At the cessation of oxidation, oxygen consumption was 6.3, 5.7, and 5.3  $\mu$ moles of oxygen per  $\mu$ mole of nicotine, 6-OHN, and 6-HPO, respectively (Fig. 4).

Crude cell extracts, which oxidized nicotine with the consumption of 2.1  $\mu$ moles of oxygen per  $\mu$ mole of nicotine, oxidized 6-HPO with the consumption of 1  $\mu$ mole of oxygen per  $\mu$ mole of substrate. The rate of oxidation of 6-HPO and nicotine were essentially identical after oxidation of the latter had proceeded beyond 0.5  $\mu$ mole of oxygen per  $\mu$ mole of nicotine (Fig. 5).

When 6-HPO was incubated with an unsupplemented 40-60 fraction, no oxygen consumption was observed but a blue pigment apparently identical to that found in the growth medium (7) was observed. If the 40-60 fraction was supplemented with brilliant cresyl blue, oxidation of 6-HPO occurred with the consumption of 0.5  $\mu$ mole of oxygen per  $\mu$ mole of substrate. In the presence of methylene blue,<sup>6</sup> the 40-60 fraction oxidized 6-HPO to various oxidation states; oxidations consuming as little as 0.5  $\mu$ mole and as much as 1.5  $\mu$ moles of oxygen per  $\mu$ mole of 6-HPO have been observed with different preparations.

After oxidation of 6-HPO by the 40-60 fraction in the presence

<sup>6</sup> Contrary to what had previously been observed (1) the 40-60 fraction prepared from most batches of cells will oxidize nicotine beyond the oxidation state of 6-HPO (1  $\mu$ mole of oxygen per  $\mu$ mole of nicotine) in the presence of methylene blue.

TABLE II

Comparison of products derived from 6-OHN oxidation and pseudooxynicotine hydroxylation

The hydroxylation of pseudooxynicotine was carried out in the following manner: 10  $\mu$ moles of pseudooxynicotine, 0.125  $\mu$ mole of methylene blue, 10  $\mu$ moles of potassium phosphate buffer, pH 7.0, and the "hydroxylating enzyme" were incubated in a total volume of 2.0 ml at 30° until oxidation ceased (0.45  $\mu$ mole oxygen per  $\mu$ mole of pseudooxynicotine). Aliquots of the reaction mixture were adjusted to the appropriate pH with HCl or NaOH as required and the absorption spectrum was determined. For chromatography, the acidified reaction mixture was spotted on Whatman No. 1 paper and developed in butanol-ethanol-water (42:42:16). The spots were visualized by spraying the paper with Dragendorff's reagent. Under these conditions, pseudooxynicotine has an  $R_F$  of 0.96.

Product source	Absorption maximum			$E_M$		$R_F$
	In 0.1 N HCl	At pH 10.6	In 0.1 N NaOH	310/289	328/310	
6-OHN.....	289	328	310	1.16	1.10	0.91
Pseudooxynicotine...	289	328	310	1.17	1.09	0.91

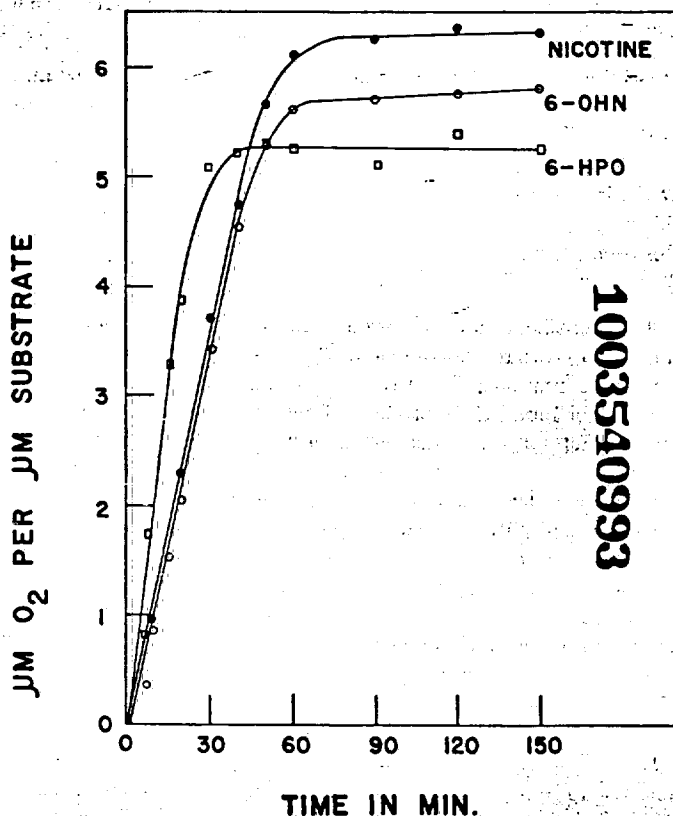


Fig. 4. The oxidation of nicotine, 6-OHN, and 6-HPO by nicotine-grown resting cells. The reaction mixtures contained the following in a total volume of 2.0 ml: potassium phosphate buffer, pH 7, 70  $\mu$ moles; 0.25 ml of an 18-hour culture of nicotine-yeast extract grown cells (strain P-34) equivalent to 731 Klett turbidity units (No. 60 filter); and either nicotine, 4  $\mu$ moles; 6-OHN, 4.5  $\mu$ moles; or 6-HPO, 1.9  $\mu$ moles as noted. Gas phase air, 30°. Data corrected for autorepiration.

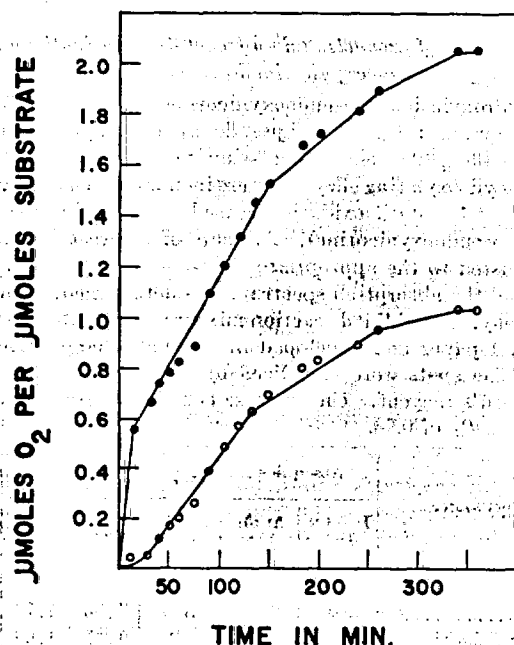


FIG. 5. The oxidation of 6-HPO and nicotine by crude cell extracts. The reaction mixtures contained the following in a total volume of 2.0 ml: potassium phosphate buffer, pH 7.0, 100  $\mu$ moles; methylene blue, 0.625  $\mu$ mole; crude enzyme, 5 mg protein; and where indicated, nicotine, 4  $\mu$ moles,  $\bullet$ — $\bullet$ ; or 6-HPO, 5.6  $\mu$ moles,  $\circ$ — $\circ$ . Gas phase air, 30°.

of brilliant cresyl blue, the reaction mixture showed a new and intense absorption maximum located at 360  $m\mu$ . Concomitantly, no Dragendorff positive material could be detected on paper chromatograms. These changes are presumably associated with the formation of the third oxidative product, the identification and isolation of which are presently being studied in this laboratory.

#### DISCUSSION

The identification of 6-HPO as a product of nicotine degradation suggests that the bacterium P-34 metabolizes nicotine by a heretofore unreported pathway. Although 6-OHN had previously been found as a product of nicotine degradation (8), its further metabolism was reported to involve an attack upon the hydroxylated pyridine ring to yield a glutamic acid derivative with an acid absorption maximum at 290  $m\mu$  which underwent a bathochromic shift to 300  $m\mu$  when the spectrum was determined in sodium hydroxide. This compound, although having absorption characteristics somewhat similar to those reported here for the product of 6-OHN oxidation, differs from 6-HPO in the following properties: the reported acid-base shift in absorption spectrum is not reversible (9); it reacts with ninhydrin; and analytical data indicate the presence of only one nitrogen in the molecule (8).

It is of interest to note that 6-hydroxymyosmine has been reported to be a product of the bacterial oxidation of nornicotine (10). This suggests that nornicotine is degraded in a manner similar to that reported for nicotine in this paper. However, since neither myosmine nor 6-hydroxynornicotine was detected, it is not clear whether nornicotine was initially hydroxylated and then dehydrogenated, or whether dehydrogenation preceded hydroxylation. Thus it is not possible to establish a formal analogy between the degradative pathways of nicotine and nornicotine, although the results are highly suggestive.

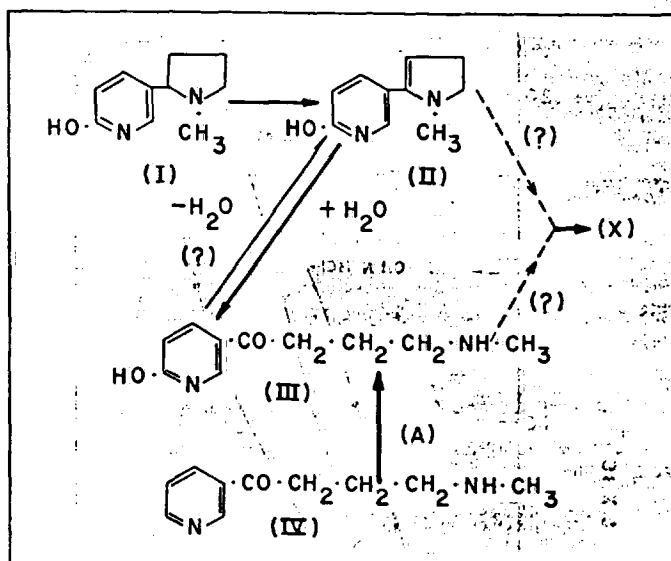


FIG. 6. Summary of reactions discussed. I, 6-hydroxynicotine; II, 6-hydroxy-N-methylmyosmine; III, 6-hydroxypseudooxynicotine; IV, pseudooxynicotine; A, hydroxylating enzyme; X, 3rd oxidative product of nicotine degradation.

The isolation of 6-HPO after the one-step oxidation of 6-OHN raises the question as to the nature of the immediate product of 6-OHN oxidation, since an examination of the structure of these compounds (Fig. 6) suggests that an intermediate exists between them. For reasons already mentioned, 6-hydroxy-N-methylmyosmine could be the intermediate. Its conversion to 6-HPO need not be enzymatic since as an  $\alpha$ -pyrroline derivative it might be expected to be hydrolyzed spontaneously under physiological conditions to 6-HPO. Thus, an analogous compound, myosmine, was reported to undergo instantaneous hydrolysis in water to 3-pyridyl- $\omega$ -aminopropyl ketone (11). The reverse type of change, cyclization of the ketone pseudooxynicotine to methylmyosmine, occurs with alkalinization (12).

It is therefore not certain whether 6-HPO is a true intermediate in nicotine degradation or whether it is formed either because 6-hydroxy-N-methylmyosmine cannot be further oxidized in the reaction system devised for its accumulation or because 6-HPO is an artifact of the purification procedure used. Although possibly not stable in the free state under physiological conditions, 6-hydroxymethylmyosmine attached to an enzyme could be sufficiently stable to allow its further metabolism without the formation of 6-HPO. These possibilities are shown in Fig. 6. That 6-HPO is oxidized by resting cells and cell-free extracts in a manner consistent with its being an intermediate in nicotine degradation is not conclusive evidence on this point since one can postulate an equilibrium between the ketone and the myosmine derivative. It is hoped that identification of compounds further along the metabolic chain will ultimately allow a decision between these possibilities.

#### SUMMARY

An enzyme fraction has been obtained from a nicotine-oxidizing soil bacterium which catalyzes the aerobic oxidation of 6-hydroxynicotine with the consumption of 0.5  $\mu$ mole of oxygen per  $\mu$ mole of substrate. The product isolated from this reaction has been identified as 6-hydroxypseudooxynicotine on the basis of elemental analysis, ultraviolet absorption spectra, and simi-

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larity to the product of pseudooxynicotine hydroxylation by an enzyme fraction which is known to catalyze hydroxylation at the six position of the pyridine moiety.

Resting cells, crude extracts, and an ammonium sulfate fractionated extract of the nicotine-oxidizing organism oxidize 6-hydroxypseudooxynicotine. Under the appropriate conditions, oxidation by the fractionated extract ceases after the consumption of 0.5  $\mu$ mole of oxygen per  $\mu$ mole of 6-hydroxypseudooxynicotine to yield a new ultraviolet absorbing substance which could be the third oxidative product of nicotine degradation.

The relation of 6-hydroxypseudooxynicotine to 6-hydroxy-N-methylmyosmine and the possible role of these compounds as intermediates in nicotine metabolism are discussed.

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April 27, 1960

**CONFIDENTIAL**The Bacterial Degradation of Nicotine and Related Compounds

The following informal summary of our investigations conducted since the preparation of the annual report for 1958-1959 is submitted as our semi-annual report. During this period we have completed our work on the third oxidative product, shown the involvement of a K- or Q-like vitamin in two of the oxidative steps studied, and obtained more information on the fourth oxidative step. Our third publication in the nicotine series is now in print (J. Biol. Chem., 235:795-80, 1960) and reprints will be sent as soon as they arrive. A report of the work on the third product will be presented at the national meeting of the Society of American Bacteriologist, May 3, in Philadelphia, and an abstract of this paper was published in Bacteriological Proceedings, p. 168 (1960).

A. The third oxidative product.

The previous annual report described the isolation in crystalline form of a compound then believed to be the third oxidative product. This compound was tentatively identified as 2,6-dihydroxy-n-methylmyosmine. Further proof of its identity has been obtained by selective reduction of the double bond in the pyrrolidine portion of the molecule by means of  $\text{NaBH}_4$ , to form a 3-substituted dipyridol. Since the substituent on the 3 position of the pyridine ring is saturated, it would not be expected to contribute significantly to the ultraviolet absorption spectrum of the reduced product, which turned out to have identical absorption characteristics to that of 2,6-dipyridol. This evidence makes conclusive the former tentative identification of the isolated product.

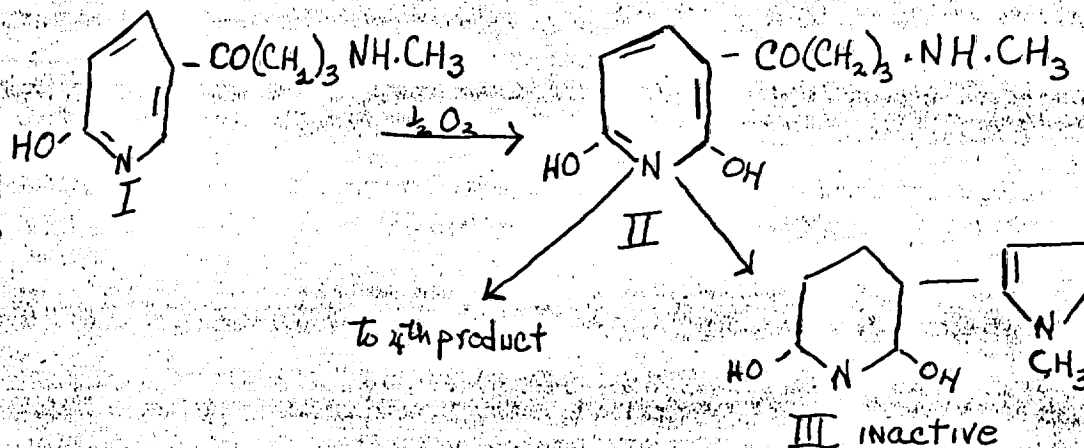
As mentioned in the previous report, the above mentioned compound was not further oxidized by our bacterium or by extracts thereof. It has now been established that this compound is actually a side product of the true third intermediate, formed by a nonenzymatic, nonoxidative, alteration of the true intermediate. When conversion of the second to third product is carried out in a spectrophotometer measuring increase in absorbance at 360 m $\mu$  (the maximum of 2,6-dihydroxy-n-methylmyosmine), two distinct rates separated by a sharp break are observed. The initial rate is very rapid and the second rate quite slow. A total spectrum run on reaction mixtures at the break point revealed a new substance present with the following properties:

1. Ultraviolet absorption maxima at 345 and 275 m $\mu$
2. A carbonyl function is present
3. The compound is irreversibly converted nonenzymatically and nonoxidatively to the previously isolated 2,6-dihydroxy-n-methylmyosmine under the conditions employed for its enzymatic formation.
4. This irreversible conversion occurs instantaneously with the application of heat or alkali.
5. Addition of acid to this material results in the formation of a compound (probably a salt) with a single absorption maximum at 320 m $\mu$ .

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6. Addition of a 0-40 ammonium sulfate fraction of the crude extract results in the rapid disappearance of its absorbancy at 345 mu.

We have as yet been unable to isolate this unstable compound which we conclude is the active third intermediate in our system. Based on the presence of a carbonyl function in the active molecule and its nonoxidative conversion to the identified dihydroxymyosmine, we propose the following sequence for the formation and transformation of the active third intermediate, 2,6-dihydroxypseudooxynicotine:



- I. 6-hydroxypseudooxynicotine (2nd product)
- II. 2,6-dihydroxypseudooxynicotine (3rd product)
- III. 2,6-dihydroxy-n-methylmyosmine

#### B. Involvement of a K- or Q-vitamin.

It has been mentioned in previous reports that extracts obtained from our bacterium fail to catalyze the first or third oxidative steps in nicotine breakdown unless they are supplemented with a redox dye such as methylene blue or brilliant cresyl blue. The nature of the cofactor being substituted for by the dye remained unknown despite numerous attempts to uncover its identity. It was recently found that several vitamin K analogues including menadione and juglone will replace the redox dyes formerly used as accessory hydrogen acceptors in these reactions. These coenzymes catalyze the reactions at rates considerably higher than those obtained using redox dyes.

Various extracts of freshly grown cells made with lipid solvents have been found to be active in this respect also. The ultraviolet spectra of these cell extracts correspond to the general pattern exhibited by coenzyme Q type compounds suggesting that such a compound is the actual cofactor. The fact that synthetic vitamin K is active does not contradict the above statement since the Q and K molecules are very similar in structure and function. If substantiated, these data establish a new function for such vitamins as being involved in hydroxylation reactions.

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C. The 4th intermediate.

Recent spectrophotometric data indicate that a new compound is formed when the 345 mμ material (II), formed in a cuvette, is subjected to the action of enzymes present in a 0-40 ammonium sulfate fraction. A new ultraviolet maximum at 325 mμ appears and in turn disappears, suggesting the temporary accumulation and further conversion of the next intermediate. The data, which is not yet conclusive, suggests a hydrolytic opening of the substituted pyridine ring to yield the 325 mμ material followed by an oxidation that destroys all ultraviolet absorption peaks.

D. Projected work.

In the next six months we hope to carry out the following investigations:

1. Further studies on blue pigment formation on which no work has been done since last summer, with special emphasis on the isolation of the pigment in pure form.
2. Isolation of the third intermediate, probably as a phenylhydrazone.
3. Attempts to isolate and identify the "Q-like" coenzyme.
4. Investigation of the fourth and fifth oxidative reactions following the pattern of work with the previous three steps.

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Renewed 10/1/57

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Activated 10/1/58

TOBACCO INDUSTRY RESEARCH COMMITTEE  
150 East Forty Second Street  
New York 17, N. Y.

Application for Research Grant

Date: June 8, 1959

1. Name of Investigator: Sydney C. Rittenberg
2. Title: Professor of Bacteriology
3. Institution &  
Address: Department of Bacteriology  
University of Southern California  
Los Angeles 7, California
4. Project or Subject: The bacterial degradation of nicotine and related compounds. The goal of the project is the elucidation of the intermediary metabolism of nicotine oxidation.
5. Detailed Plan of Procedure: The program and progress of this investigation have been detailed in past grant applications, in semi-annual and annual reports, and in the two papers published to date. On the assumption that the above material is part of the record, only a brief statement of the work contemplated for next year is included here.

It is our intent to continue the research along the lines of the past three years. The identification of the third oxidative product, which we now have in crystalline form, is currently receiving most attention. We have recently worked out the conditions necessary for the accumulation of the fourth product and a chromatographic technique for its detection. We will shortly attempt a large scale (several hundred milligrams) enzymatic synthesis of this compound and start purification and identification studies as was done for the three preceding metabolic intermediates. We will investigate the conditions necessary for the accumulation of the fifth oxidative product. Since our enzyme preparations oxidize nicotine beyond this point, and since the chromatographic technique worked out for the fourth compound may also apply to the fifth, the prerequisites for this phase of the work are completed.

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Finally, over the course of last year we have obtained, by isolation and from other investigators, a collection of about a dozen different nicotine oxidizing bacteria. We plan to investigate the ability of these organisms to metabolize the intermediates of nicotine oxidation we have isolated using the simultaneous adaptation technique. From such studies we should get some information as to whether the pathway of nicotine oxidation carried out by our organism is a general one. It is improbable that more than the above, if that much, will be completed within the next year.

6. Budget Plan:	Salaries	\$4,800.00
	Expendable Supplies	1,500.00
	Permanent Equipment	
	Overhead	532.00
	Other = Travel	350.00
	Total	\$7,182.00

7. Anticipated Duration of Work: One year for work outlined. About two additional years to complete the project.

8. Facilities and Staff Available: The facilities of a well equipped bacteriology-biochemistry laboratory are available and no additional needs are anticipated.

The staff will consist of the director of the project and two graduate students. One, Mr. S. Richardson, has advanced to candidacy for the Ph.D. degree and will be working essentially full time on the project. He will receive \$3600 for the year. The second student, not yet chosen, will be working only part-time, and will be used mainly to assist in organic syntheses and other chemical aspects of the program.

9. Additional Requirements: None.

10. Additional Information (Including relation of work to other projects and other sources of supply):

The relation of our work to projects in progress elsewhere and to current knowledge of nicotine metabolism has been discussed in previous grant applications and in the semi-annual and annual reports, and there is little new to mention here.

University of Southern California  
University Park  
Los Angeles 7, California

Signature /s/ Sydney C. Rittenberg  
Director of Project

/s/ Elton D. Phillips  
Business Officer of the Institution

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Grant # 209  
& 86

P13. *The hydroxylation of myosmine, nornicotine, and pseudooxynicotine.* LAWRENCE I. HOCHSTEIN and SYDNEY C. RITTENBERG, Department of Bacteriology, University of Southern California, Los Angeles.

Previous studies have established that an enzyme fraction, obtained from a nicotine-oxidizing soil bacterium, hydroxylates nicotine, with the consumption of 0.5  $\mu$ mole of oxygen per  $\mu$ mole of substrate, yielding 6-hydroxynicotine. Manometric experiments showed that the hydroxylating enzyme fraction did not oxidize pyridine, nicotinic acid, nicotinamide, anabasine, or 3-nicotinylpropionic acid. Myosmine, nornicotine, and pseudooxynicotine were oxidized: 0.5  $\mu$ mole of oxygen per  $\mu$ mole of substrate was consumed during the reaction. It would appear that the structural requirements of the hydroxylating reaction involve more than the pyridine moiety of nicotine. Whether the intact pyrrolidine ring is a structural requirement is more difficult to assess. The oxidation of nornicotine and myosmine would indicate as much. However, it is not certain whether myosmine is oxidized as such, or as its open, hydrated form. A similar uncertainty exists with respect to pseudooxynicotine.

The product of myosmine oxidation had a single absorption maximum, at 298  $m\mu$  in 0.1N HCl and at 280  $m\mu$  in 0.1N NaOH. These absorption characteristics correspond to those reported for synthetic 6-hydroxymyosmine. The ultraviolet absorption spectra of the products of nornicotine and pseudooxynicotine oxidation, as determined in reaction mixtures, also suggests that these compounds are hydroxylated at the six position by the enzyme. The product of pseudooxynicotine oxidation is identical in absorption characteristics to a product formed by a two step enzymatic oxidation of nicotine. This and other data identifies the second oxidative produce of nicotine metabolism as 6-hydroxy-N-methylmyosmine or its hydrated form.

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TOBACCO INDUSTRY RESEARCH COMMITTEE

150 EAST FORTY SECOND STREET NEW YORK 17, N.Y.

Application for Research Grant

Date: July 16, 1959

1. Name of Investigator: Benson B. Roe, M.D. and Leon Goldman, M.D.  
Associate Professor of Surgery Professor and Chairman
2. Title: and Chief, Cardiac Surgery Department of Surgery
3. Institution School of Medicine  
& Address: University of California  
3rd and Parnassus  
San Francisco 22, California
4. Project or Subject: The Action of Negatively Charged Ions on Tracheo-Bronchial Ciliary Action in the Human Patient.
5. Detailed Plan of Procedure: In vitro studies on animal trachea by Doctor Albert P. Krueger, Professor of Bacteriology, Emeritus, at the University of California, and living animal studies on tissue susceptibility to trauma have indicated significantly detrimental effects of positively charged ions in the inhaled air. Negative ions have been demonstrated to produce increased ciliary action and to promote tracheal cleansing. Little work on this subject has been done in this country though extensive studies have been reported in Europe. The potential clinical value of this work is significant and of particular importance is its use in the postoperative patient to aid mucous evacuation and avoid atelectasis.

Method: Safe, effective air negative ionization apparatus has been developed by the Wesix Electric Company in San Francisco. Using this apparatus or modifications of it, a combined clinical-pathological study is planned.

- A. Clinical studies will utilize a double blind method of evaluating postoperative respiratory complications in patients who will be exposed to a negative ion atmosphere. It is planned to use a group of goose-necked ionizers at the patient's bedside, some of which are dummies not identifiable to those making the clinical evaluation.

Negative ion apparatus may be rigged into anesthesia machines and it is planned to carry out this work in conjunction with Dr. Stuart Cullen in the Anesthesia Department to evaluate clinically the effect of negative ionization during anesthesia. It is significant that high pressure with turbulent flow (such as anesthesia machines and air conditioning) produces positive ionization in the very areas where it is theoretically most detrimental.

1003541002

B. Pathological studies: Microscopic quantitative measurement of ciliary action will be studied in a constant temperature negative ion atmosphere using a stroboscopic light. Specimens of freshly resected human bronchi will be obtained from the operating theater in this Hospital and elsewhere to verify the human application of animal studies performed by Dr. Krueger. After establishing the ciliary response of bronchial mucosa with controlled ionization in the laboratory, it is planned to study the differential effect on ciliary action of various anesthetic agents with and without negative ions in the anesthetic apparatus.

Approximately 3,000 general anesthetics are given annually in the University of California Hospital. It is planned to begin with a pilot clinical study on the Thoracic Surgical Service to establish well-defined criteria for the definition of "pulmonary complication". Thereafter it is planned to study a wide variety of surgical patients, subjecting all patients included in this series to identical negative ion "treatment". Approximately one-half of the patients will be "treated" with dummy ionizers. For a statistical validity of this comparative evaluation it will probably be necessary to study several hundred patients.

6. Budget Plan:	(One full-time	
	Salaries (senior technician**	\$ 4,980.00
	Expendable Supplies	500.00
	Permanent Equipment	1,700.00
	Overhead (15%)	1,092.00
	Other (Transportation to obtain specimens)	100.00
		<hr/> \$ 8,372.00

7. Anticipated Duration of Work: Two to three years.

8. Facilities and Staff Available: Pathological Laboratory and clinical services of the University of California Hospital.

9. Additional Requirements: None.

10. Additional Information (Including relation of work to other projects and other sources of supply):

Pilot studies have been supported by the Committee on Research of our School of Medicine.

Make check payable to The Regents of the University of California.

\*\* It is anticipated that a full-time technician will be needed to maintain and operate the apparatus for counting ciliary action in the surgical specimens. Also, this technician will aid in the gathering and tabulating of the clinical data.

/s/ Benson B. Roe, M.D.  
Director of Project

/s/ James H. Corley  
Vice President-Governmental Relations  
and Projects

1003541003



**CONFIDENTIAL**

TIRC Grant #250

Progress Report #1

Benson B. Roe, M.D.  
University of California  
School of Medicine

July 8, 1960

The Action of Negatively Charged Ions on Tracheo-Bronchial Ciliary Action  
in the Human Patient

---

In reply to your letter of July 5, this letter is to provide your committee with an interim progress report on our project to date. We are grateful for your reconsideration of additional support as requested and it will be possible for us to await the decision of the committee on September 22 without major inconvenience.

I regret to say that we are unable to report spectacular success up to the present, but considering the problems in obtaining the special equipment necessary and of learning the difficult techniques involved with new personnel, I see no cause for discouragement. Because of repeated efforts over the past three years to carry out this project with resident personnel or research fellows engaged in other projects, I became convinced that the successful accomplishment would depend upon the full-time concentration of an intelligent technician who could learn the methods, master the techniques, and be constantly on hand for tissues when they become available. Since the techniques were those with which no one could have had previous experience, we of necessity hired a technician "blind." By good fortune we have obtained a very industrious, intelligent, resourceful young lady who immediately acquainted herself with the basic techniques as developed and described by Dr. Krueger in his laboratory at Berkeley. She then reviewed with me in great detail the available literature on ciliary action and air ionization.

During the several weeks it required to obtain the necessary equipment for air ionization and ciliary action study, she became familiar with the other research activities in the laboratory which has proved excellent background for her present work.

After obtaining stroboscopic light and microscope for studying ciliary action, we unfortunately wasted several weeks in attempts to obtain reliable measurements of ciliary speed. When finally it was discovered that the stroboscopic light was working improperly; a substitute light was obtained and now excellent data is being recorded. The observations are now sufficiently reproducible and her technique is sufficiently skillful that we can now begin in earnest to study human surgically resected bronchial mucosa to substantiate the animal studies already reported.

Ion-counting apparatus is now being used to determine the degree of ionization of hospital environmental air, including anesthesia machines, recovery room wards, and oxygen tents. Pilot models of plastic helmets to control the respiratory atmosphere of postoperative patients have been made and it is anticipated that ionization in these helmets can be controlled.

1003541004

Ciliary action of tobacco smoke atmosphere will be measured and correlated with ionization control. Ciliary action in human bronchial specimens will also be correlated with history of tobacco smoking.

The Committee's support of this work is greatly appreciated and satisfactory progress to date suggests a rewarding outcome.

- XXX -

1003541005

## Committee:

## TOBACCO INDUSTRY RESEARCH COMMITTEE

Drs. Kotin, Chm.

Jacobson

Reimann

Rienhoff

150 East Forty Second Street

New York 17, N.Y.

Activated: 3/1/60

Application for Supplementary FundsDate: July 28, 1960

1. Name of Investigator: Benson B. Roe, M.D.  
Leon Goldman, M.D.
2. Title: The Action of Negatively Charged Ions on Tracheo-Bronchial Ciliary Action in the Human Patient.
3. Institution & Address: University of California Medical Center  
San Francisco 22, California
4. Project or Subject: In vitro studies on animal trachea by Dr. Albert P. Krueger (Professor of Bacteriology, Emeritus, University of California) and living animal studies on tissue susceptibility to trauma have indicated significantly detrimental effects of positively charged ions in the inhaled air. Negative ions have been demonstrated to produce increased ciliary action and to promote tracheal cleaning. Little work on this subject has been done in this country, though extensive studies have been carried out in Europe. The potential clinical value of this work is significant, and of particular importance in its use in the postoperative patient to aid mucous evacuation and avoid atelectasis.
5. Detailed Plan of Procedure:

Method:

Safe, effective air-negative ionization apparatus has been developed by the Wesix Electric Company in San Francisco. It is planned to carry out a long-term study of clinical effectiveness of this measure in the postoperative patient both during and after anesthesia. In vitro studies on fresh surgical specimens of human bronchus will be carried out simultaneously on this campus to determine whether ciliary activity and bronchial trauma are effected by positive and negative ions in the same manner as that demonstrated by Dr. Krueger's animal studies.

6. Budget Plan:

a. Salaries	-----
b. Expendable Supplies	\$ 100.00
c. Permanent Equipment	750.00
d. Overhead (15% of a / b)	15.00
e. Other	-----
	\$ 865.00

1003541006

7. Anticipated Duration of Work: Two or three years.
8. Facilities and Staff Available: Benson B. Roe, M.D. (Assoc. Prof. of Surgery)  
Henrietta Chandler, B.S. (Laboratory Technician)  
Laboratory is located in the Experimental Surgery Building.  
Equipment already purchased includes 1) Dissecting microscope, 2) AIR Ion target collector, 3) Wesix Mark IV Ion collector, 4) Vibrating Reed type micro-micro-ammeter, 5) Strobotac Light.
9. Additional Requirements: Additional financial support is requested. Estimates of various costs in the categories listed have already proven to be unrealistic so far as our original grant request was concerned. The continuation of the project is dependent upon further support not available from existing local funds or facilities.

Expendable Supplies:

Original request was for \$500. In the first three months of operation the expendable supplies have been for \$126.80. Further supplies have been already requisitioned.

Permanent Equipment:

Original request was for \$1,700 based on available catalog costs of necessary equipment as listed. These costs have all exceeded the original estimates and before completing our equipment even for the basic studies, without including that for the clinical studies, a total of \$2000 has been spent.

10. Additional Information (Including relation of work to other projects and other sources of supply):

/s/ Benson B. Roe  
Director of Project

/s/ Stanley C. Bateman  
Business Manager

1003541007



TOBACCO INDUSTRY RESEARCH COMMITTEE

TOBACCO INDUSTRY RESEARCH COMMITTEE

150 East Forty Second Street

New York 17, N.Y.

#231

Application for Research Grant

Date: February 28, 1959

1. Name of Investigator: Leslie Michael Rosa, M.D.<sup>1</sup> and Aldo A. Luisada, M.D.<sup>2</sup>
2. Title: (1) Assistant Professor of Medicine - (2) Assoc. Prof. of Medicine;  
Dir., Division of Cardiology
3. Institution & Address: The Chicago Medical School, Division of Cardiology,  
2755 W. 15th St., Chicago 8, Illinois
4. Project or Subject: Investigation on the effect of cigarette smoking in 50 normal subjects and 50 patients with coronary heart disease with special emphasis on records of precordial displacement and acceleration (a 2 year study).
5. Detailed Plan of Procedure (Use reverse side if additional space is needed):

Has cigarette smoking any effect on the cardiovascular system?

The significance of objective methods in the verification of similar findings is unquestionable. Records of precordial displacement and acceleration in the filtered ballistic frequency range represent objective criteria for changes in circulatory dynamics (L. M. Rosa: Einführung in die ballistische Kardiographie, Edition Regensburg, Muenster, 1958; in press: Amer. J. of Cardiology).

Precordial displacement corresponds to the lowest frequency range of vibrations caused by the heart beat (0-5 cycles per second). Precordial acceleration is the second mathematic derivative of displacement. It may be registered by the use of accelerometers, mathematic or graphic differentiation of the displacement tracing, or the application of band pass filters in the range 5-25 c.p.s. Both precordial displacement and acceleration tracings have been studied in basic experiments by the investigators. These tracings have been found closely related to total body ballistocardiograms; however, pattern and time relationship to circulatory events seem to be more constant and reproducible in precordial derivation. Standard patterns have been used as the basis of comparison with conditions of changed cardiovascular dynamics, and significant alterations have been found.

Preliminary tests carried out by Dr. Rosa, by using this method, failed to disclose significant effects produced by smoking within 15 minutes. This observation requires a much more extensive study for evaluation, but is sufficient to awaken new interest in the problem.

The investigators are going to conduct a two-year research program in coronary heart disease under the sponsorship of the American Heart Association. This study will include the workup of 50 normal subjects and 50 patients suffering from coronary heart disease.

Apart from precordial methods, routine electrocardiography, phonocardiography, chest x-ray, ballistocardiography, and usual clinical tests will be made for 2 subsequent years. It is suggested that, under the sponsorship of the Tobacco Industry Research Committee, a parallel study be made. The effect of cigarette smoking would be studied on the same clinical material, including both normal subjects and coronary patients.

Buechley and coworkers (Circulation, 18: 1085, 1958) from the California State Department of Public Health, found a significant difference in death rate from coronary heart disease between non smokers and heavy smokers. The study has been based on 19 similar previous publications.

A research group at Johns Hopkins University published five experimental reports on this problem and stated (F. M. Davis: The Am. J. of Cardiology, 3:103, 1959) that changes in cardiovascular dynamics in patients with coronary heart disease are dramatically reflected in the ballistocardiogram after the routine smoking of a cigarette.

The present study, as stated above, will compare, among other data, the low frequency tracing of the chest wall with the ballistocardiogram of the body. If any change in tonus of the skeletal muscle is responsible for BCG changes, the low frequency tracing of the chest, which is not influenced by muscular tonus, will provide an objective basis for the explanation of the previous findings.

Budget

1/3 salary of a full time Assistant	\$2,000
1/2 salary of a full time technician	2,500
1/4 salary of a full time secretary (reports and filing)	1,750
photographic supplies	300
travel, meetings, books	300
8% overhead	548

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\$ 7,398 per year

A Progress Report of the work accomplished will be sent to The Tobacco Industry Research Committee on July 1 of each year.

1003541009

TOBACCO INDUSTRY RESEARCH COMMITTEE

TOBACCO INDUSTRY RESEARCH COMMITTEE

150 East Forty Second Street

New York 17, N.Y.

#231

Application for Research Grant

Date: February 28, 1959

1. Name of Investigator: Leslie Michael Rosa, M.D.<sup>1</sup> and Aldo A. Luisada, M.D.<sup>2</sup>
2. Title: (1) Assistant Professor of Medicine - (2) Assoc. Prof. of Medicine;  
Dir., Division of Cardiology
3. Institution & Address: The Chicago Medical School, Division of Cardiology,  
2755 W. 15th St., Chicago 8, Illinois
4. Project or Subject: Investigation on the effect of cigarette smoking in 50 normal subjects and 50 patients with coronary heart disease with special emphasis on records of precordial displacement and acceleration (a 2 year study).

5. Detailed Plan of Procedure (Use reverse side if additional space is needed):

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Budget

1/3 salary of a full time Assistant	\$2,000
1/2 salary of a full time technician	2,500
1/4 salary of a full time secretary (reports and filing)	1,750
photographic supplies	300
travel, meetings, books	300
8% overhead	548
	<hr/>
	\$ 7,398 per year

A Progress Report of the work accomplished will be sent to The Tobacco Industry Research Committee on July 1 of each year.

1003541011

TOBACCO INDUSTRY RESEARCH COMMITTEE  
150 EAST FORTY SECOND STREET  
NEW YORK 17, N.Y.

181

Application For Research Grant

Date: January 6, 1958

1. Name of Investigator: Dr. Benjamin A. Rubin
2. Title: Assistant Professor - Department of Public Health and Preventive Medicine
3. Institution & Address: Baylor University College of Medicine, 1200 M. D. Anderson Blvd., Houston 25, Texas

4. Project or Subject:

An evaluation of the phenomenon of tumor growth enhancement as an assay for carcinogens among the polycyclic hydrocarbons and related compounds.

5. Detailed Plan of Procedure (Use reverse side if additional space is needed):

We have noted (1,2,3) that when carcinogenic polycyclic aromatic hydrocarbons are applied to the skin of certain strains of mice, they are thereafter able to support the progressive growth of a tumor to which they are normally resistant. The non-carcinogenic analogs tested did not have this effect and weakly carcinogenic agents of related structures showed intermediate effects.

Enhancement could be measured in three ways, 1) The fraction of animals showing progressive tumor growth, 2) The extent of growth of the tumors that eventually regress, and 3) The time of persistence of the tumor after controls regress. It was thus possible to devise quantitative, as well as a qualitative estimation of tumor growth enhancement. The plan of procedure for evaluating the usefulness of this effect as an assay method would include the following work.

A group of polycyclic hydrocarbons of analogous series would be synthesized, such that there is a known progression of carcinogenicity, both in terms of total effectiveness and in variations of latency in the classical tests. Such series exist in the methylated benzantracenes, and in the substituted benzimidines (4,5). We have available the services of a synthetic organic chemist who is able to prepare these compounds.

1003541012



These series would first be compared in susceptible mice, in a standard fashion in which 0.5 micromols in benzene are dropped on the shaved skin of two groups of six mice each 1) six times in two weeks 2) 18 times in six weeks (every Monday, Wednesday and Friday). Compounds that produce more than 33% of progressive growth in six paintings would be suitably diluted and reapplied for six times at various diminishing concentrations.

Although we have been able in this manner (18 times) to detect a significant effect in the very weak carcinogen 1:2 benzantracene--if any compound fails to show perceptible effect it will be reapplied for 18 times at increasing levels up to 5.0 micromols per application (solubility permitting).

As far as possible these compounds will then be graded according to the total amount required to produce 33% (estimate 37%) progressive growth. This will be compared with the published (carcinogenicity) values given in the literature for the same substances from classical test methods (5).

In view of the demonstrations that weak carcinogens may inhibit the effect of strong ones, among the polycyclic hydrocarbons, similar quantitative experiments will be carried out, using combinations of agents which have been shown to be "competitive." And we would also test mixtures which have been reported to be additive (6).

We would also determine the quantitative effect in this system of strong carcinogens, such as 3,4 benzpyrene (which is strongly tumor enhancing) alone and in the presence of hydrocarbon mixtures such as composite cigarette smoke condensate (Ecusta) and the benzene-soluble fraction of industrial air pollutants. Effects of each of these mixtures alone would also be found. It might thus be possible to determine whether naturally occurring complex hydrocarbon mixtures can interfere with the detectability of known carcinogens.

This approach gives promise of providing a rapid, economical, and quantitative method of detecting the important carcinogens of the polycyclic hydrocarbon group, even when they exist as minor impurities in complex mixtures. In view of the time requirements and theoretical complexities of present methods for carcinogen testing, it seems advisable to explore the utility and limitations of this enhancement phenomenon. Should it prove to correlate well with classical carcinogenesis studies, it would greatly facilitate the isolation of small active constituents in the complex mixtures obtained from tobacco or air pollution materials.

#### References

1. Rubin, B.A., Ida N., and Kirschbaum, A. Growth Enhancement of a Transplanted Mouse Lymphosarcoma. *Proc. Cancer Research*, 2:143 (1956).
2. Rubin, B.A., Ida N., Studies on the mechanism of growth enhancement of transplanted mouse tumors by carcinogens. *Proc. Cancer Res.* 2:244 (1957).
3. Rubin, B.A. and Ward, E.B. A new genetic factor affecting tumor transplantation in mice. *Genetics* 42:392 (1957).
4. Badger, G.M., *Chemical Constitution and Carcinogenic Activity in Advances in Cancer Research*. Vol 2, Academic Press N.Y. 1954.
5. Greenstein, J.B. *Biochemistry of Cancer* Academic Press NY 1954.
6. Steiner, P.E. and Falk, H.L. Summation and inhibition effect of weak and strong carcinogenic hydrocarbons. *Cancer Res.* 11:56 (1951)

1003541013

6. Budget Plan:

Approximately \$2000 is for chemical synthetic work; \$700 for chemicals and supplies and \$1800 for animals.

Salaries  
Expendable Supplies  
Permanent Equipment  
Overhead 15%  
Other

	0
	<u>\$4,500</u>
	0
	<u>675</u>
	0
Total	<u>\$5,175</u>

7. Anticipated Duration of Work:

January 1, 1958 to December 31, 1958

8. Facilities and Staff Available:

We have the use of equipped biological and chemical laboratories and adequate animal quarters. The staff available for this work includes the senior investigator (B.A. Rubin) and Miss Margaret Stitt, research assistant.

9. Additional Requirements:

We will utilize as a consultant, Dr. Thomas Odene, a synthetic organic chemist, of considerable experience in this field. He has agreed to prepare the needed compounds (not commercially available) for an appropriate fee.

10. Additional Information (Including relation of work to other projects and other sources of supply):

We have another grant in the field of Cancer Immunology from the National Cancer Institute for about \$17,500 a year. This is used in part to elucidate the relation of the enhancement phenomenon to the mechanisms of host response to foreign tissue and tumors.

1003541014

Dean, Stanley W. Olson, M.D.

Signature s./ B. A. Rubin  
Director of Project , B. A. Rubin

/s./ Alfus O. Johnson  
Business Officer of the Institution , Alfus O. Johnson



TOBACCO INDUSTRY RESEARCH COMMITTEE  
150 EAST FORTY SECOND STREET NEW YORK 17, N. Y.

#18181

(Activated 1/1/58)

Application For Research Grant

Date: November, 1958

1. Name of Investigator:

B. A. Rubin, Ph.D.

2. Title:

Assistant Professor of Epidemiology

3. Institution

& Address:

Department of Epidemiology  
Baylor University College of Medicine  
Texas Medical Center  
Houston 25, Texas

4. Project or Subject:

The comparison of the effects of carcinogenic polycyclic hydrocarbons on tumor induction with tumor enhancement.

5. Detailed Plan of Procedure (Use reverse side if additional space is needed):

Please refer to original application #181 - dated 1/6/58.

1003541015

6. Budget Plan:

Remodeling of animal rooms	\$3000	Salaries	
Animal cages and racks	2000	Expendable Supplies	
Animal care and food	3000	Permanent Equipment	
Overhead (15%)	1200	Overhead	
	<u>\$9200</u>	Other	

Total \$ 9,200

7. Anticipated Duration of Work:

**One year**

8. Facilities and Staff Available:

9. Additional Requirements:

10. Additional Information (Including relation of work to other projects and other sources of supply):

/s/ J. L. Malnick, Ph.D.  
Chairman, Dept. of Epidemiology

/s/ Stanley W. Olson, M.D., Dean

Signature /s/ A. A. Rubin, Ph.D.  
Director of Project

/s/ Stanley W. Olson  
Business Manager of Institution

**Business Manager**

1003541016

TIRC Grant #181

Benjamin A. Rubin, Ph.D.  
Baylor University**CONFIDENTIAL**

Report No. 1

November 14, 1958

An Evaluation of the Phenomenon of Tumor Growth Enhancement  
as an Assay for Carcinogens Among the Polycyclic  
Hydrocarbons and Related Compounds

Dear Dr. Hockett:

Thank you for your letter of October 28. I was wondering what you would want in the way of a report. Your request is a very good answer to my question.

It is difficult to say much about this project without referring to the details of a rather complex background. This I would like to provide in the form of my report to the U.S. Public Health Service, which is enclosed. As you can see, it covers the work through the first half of this year.

I would like to point out especially the sections on: 1) Effects of carcinogens on tumor growth -- page 5; 2) Quantitation of carcinogen enhancement effect -- page 6; 3) Effect of other carcinogens and related hydrocarbons -- page 7; 4) Transplantation of skin -- page 12; and 5) Studies in chemotherapy -- page 14. All of these refer to some newer aspects of the effect of carcinogens on transplantation.

In addition to the material in these sections, we are doing work on the standardization of the method. Because of occasional variation in the extent of tumor growth in normal mice -- and the consequent variation in "controls", against which our effect must be measured -- we conducted (and continue) some studies on the growth of different tumor lines in C3H, CBA, DBA/2, and various hybrids. In brief, the behavior of the tumor in DBA/2 mice seems to depend upon the mice in which it was previously carried. We have tested three tumor (6C3HED) lines from Rutgers and three of our own in a variety of situations. It is too early to go into much detail, except to say that the tendency is for the controls to diverge more from carcinogen treated animals. It has been occasional contention of Dr. Hauschka that "anything" grows in DBA mice -- and therefore, this effect is not surprising. Our findings would indicate that you have to control conditions carefully to get this tumor to grow at all in these mice. While this may provide a more clear-cut effect for strong carcinogens, it cuts down on sensitivity for weak ones.

Since the compounds we want to test are so valuable (and scarce), I have hesitated to let them go out until the test could provide reliable and reproducible quantitative effects. The alternative is to test everything at the same time -- which would require an immense number of mice. We are indeed building up toward a larger group, but I trust this other problem will soon be resolved.

It may be of interest to you to know that Dr. Fred Bock has written to me recently about his work with this system. He complains that his growth in DBA mice is too slow -- although he easily confirms the basic findings. I have written to him about my observations on the tumor and its origins. He is experiencing another aspect of this same problem.

1003541017

The other part of our work concerns the synthesis and procurement of carcinogens and their analogs. With the help of Dr. Osdene we have synthesized good quantities of six compounds and will probably have 2-3 more before the end of the year. These are all benzacridines of varying levels of carcinogenicity. We have also purchased 10 additional members of this series from the Light Company in England.

In addition, we have obtained a large group of methylated benzanthracenes, benzphenanthrenes, benzpyrenes, crysenes, and cholanthrenes.

Many of these are available through the collaboration of Dr. Ralph S. Becker of the University of Houston. He has a wonderful collection of high purity standards for his work on spectral properties. He is eager to find a reliable biological test to compare with his physical findings. He was referred to me by the N.C.I., and we should both profit.

We will compare the physical properties of these compounds with  
1) a standardized classical technique (32 weeks of skin painting), and  
2) the tumor enhancement effect.

This will permit me to make my own comparisons of effect, rather than to rely upon the data in a literature of unknown variability.

Becker has found a very good correlation between the energy difference between two electronic absorption band maxima and published carcinogenesis. Other emission characteristics (fluorescence, phosphorescence, etc.) are providing additional information of value. But the biological data lack the quantitativeness of his observations -- and we hope that I can provide such quantitation with the present study.

To sum up, we have made progress in several directions, but this study will take longer than I had anticipated. However, the present developments indicate that we may come out with more valuable data than was previously possible.

If you require more details of my data or Becker's findings, I will be happy to provide them.

-----

1003541018

C O P Y

BAYLOR UNIVERSITY  
COLLEGE OF MEDICINE  
TEXAS MEDICAL CENTER  
HOUSTON, TEXAS

September 30, 1957

Department of Public Health and Preventive Medicine

Dr. Robert C. Hockett  
Associate Scientific Director  
Tobacco Industry Research Committee  
150 East Forty Second Street  
New York 17, New York

Dear Dr. Hockett:

I am writing to you at the suggestion of Dr. Harry Heimann, Chief of the Operational Research Section, Air Pollution Medical Program of the U. S. Public Health Service. That organization has been interested in our cooperation in their programs. These were to be organized in such a way that our studies were to be closely coordinated with those of Dr. W. C. Hueper, Chief of the Environmental Cancer Section of the National Cancer Institute.

I am enclosing a copy of our application to the Public Health Service for the project suggested. Because of some administrative problems this project can not be supported until next spring at the earliest by the Public Health Service. Since the Air Pollution Program would like to see us take up our part of the work immediately it was suggested that your organization might be interested in support of this program until the Public Health Service can take over. The copy of our application should explain to you the matter of the proposed study. If this falls within your realm of interest and there is a possibility of such support I would be happy to supply you with whatever additional information you might require.

It might be of some interest to know that we have progressed considerably beyond the work described and have tested several complex mixtures including some from tobacco extracts. The method described has been shown to be capable of revealing minute amounts of carcinogens (polycyclic hydrocarbons) even in the presence of a large amount of inert carrier.

I look forward to hearing from you on this matter.

Very sincerely,

/s/ B. A. Rubin  
Benjamin A. Rubin

1003541019

(Leave Blank)  
Council assigned May 1957  
Action May 1957

Department of  
HEALTH, EDUCATION, AND WELFARE  
PUBLIC HEALTH SERVICE  
NATIONAL INSTITUTES OF HEALTH  
**APPLICATION FOR RESEARCH GRANT**

(LEAVE BLANK)  
C-3666  
S.E.O.H. (1)  
Former grant No.             
If different           

PRIVILEGED COMMUNICATION  
PUBLIC HEALTH SERVICE  
NATIONAL INSTITUTES OF HEALTH  
DIVISION OF RESEARCH GRANTS  
Bethesda 14, Maryland

Rec'd 5-24-57

Date May 20, 1957

Application is hereby made for a grant in the amount of \$ 31,202.00  
from May 20, 1957 through May 19, 1958  
Month Day Year Month Day Year  
Inclusive for the purpose of conducting a research project on the following subject:

(LIMIT TITLE TO 53 LETTERS AND SPACES)  
Title Of Project Tumor Enhancing and Carcinogenic Effects of Air Pollutants Correlated with Human Cancer Epidemiology

Name, Title And Address Of Principal Investigator  
Dr. Hardy A. Kemp, Chairman  
Department of Public Health  
Baylor University College of Medicine  
Dr. Benjamin A. Rubin, Asst. Professor  
Department of Preventive Medicine  
Baylor University College of Medicine  
Houston 25, Texas

Names And Titles Of Co-investigators, If Any  
Dr. Arthur Kirschbaum, Chairman  
Department of Anatomy  
Mr. Alan C. Love, Chief Engineer  
City of Houston Health Department  
Dr. Thomas S. Oedene, Asst. Biochemist  
Univ. of Texas M. D. Anderson Hospital  
and Tumor Institute

Name Of Financial Officer  
To Whom Check Should Be Mailed  
Alfus O. Johnson

Title And Address Of Financial Officer  
Business Manager  
Baylor University College of Medicine  
Houston 25, Texas

Check Should Be Made Payable To:  
Baylor University College of Medicine

Page 3 omitted - no entries. (R) **AGREEMENT**

It is understood and agreed by the applicant: (1) That funds granted as a result of this request are to be expended for the purposes set forth herein; (2) that the grant may be revoked in whole or part at any time by the Surgeon General of the Public Health Service, provided that a revocation shall not include any amount obligated previous to the effective date of the revocation if such obligations were made solely for the purposes set forth in this application; (3) that all reports of original investigations supported by any grant made as a result of this request shall acknowledge such support; (4) that, if any invention arises or is developed in the course of the work aided by any grant received as a result of this application, the applicant institution will either (a) refer to the Surgeon General for determination, or (b) determine in accordance with its own policies, and subject to the terms of its agreement with the Surgeon General dated           , whether patent protection on such invention shall be sought and how the rights in the invention, including rights under any patent issued thereon, shall be disposed of and administered, in order to protect the public interest.

NAME OF INSTITUTION Baylor University College of Medicine

NAME AND TITLE OF  
OFFICIAL AUTHORIZED  
TO SIGN FOR INSTITUTION  
(Please Type) Stanley W. Olson, Dean

PERSONAL SIGNATURE Stanley W. Olson M.D.  
(This agreement must carry the actual signature of the official whose name appears on the line above.)

PAGE 1

PRIVILEGED COMMUNICATION

Form Approved  
Budget Bureau No. 68-R249.5

1003541020

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C-3666

PROPOSED BUDGET, for the period shown on page 1

NOTE: Under column entitled "OTHER" indicate funds presently available or anticipated from other sources including own institution.

## BUDGET

	REQUESTED FROM PHS (omit cents)	OTHER
PERSONNEL (ITEMIZE ALL POSITIONS BY INDICATING TYPE, NAMES OF PROFESSIONAL PERSONNEL, IF SELECTED)		
Dr. B. A. Rubin	REDACTED	
Chemist		
Laboratory Assistant		
Animal Caretaker, half time		
PERMANENT EQUIPMENT (Itemize)		
High Velocity Equipment	1,000.00	
Gas Sampling Equipment	1,000.00	
Air Flow Measurement and Calibration Equipment	1,000.00	
Animal cages, 3 racks	1,500.00	
CONSUMABLE SUPPLIES (Itemize)		
Animals, feed, laboratory supplies, reagents, filters, etc.	3,000.00	
TRAVEL (State Purpose)		
Scientific Meetings and Consultations in other laboratories	1,000.00	
OTHER EXPENSE (Itemize)		
Transportation for sampling equipment	1,000.00	
Chemical consultations and Commercial analysis	3,000.00	
Equipment maintenance	500.00	
Social security and other	500.00	
	<b>SUBTOTAL (Direct Costs)</b>	
	\$ 27,150.00	
	<b>PHS PARTICIPATION IN INDIRECT COSTS (omit cents — adjust to low dollar)</b>	
	4,052.00	
<b>TOTAL BUDGET (omit cents)</b>		
\$ 31,202.00		

NOTE: The administrative official signing this application may add an amount for indirect costs in accordance with the instructions.

ESTIMATE OF FUTURE REQUIREMENTS—applies to funds needed from the Public Health Service for the years subsequent to the period proposed for this application. The spaces at the right are to be used to indicate the amount of support needed for each year, showing the direct and indirect costs as appropriate. DO NOT LEAVE ANY SPACES BLANK—if no additional support is required, enter "None". FOR FURTHER INFORMATION: See detailed instructions accompanying application form.

	DIRECT COSTS	INDIRECT COSTS	TOTAL
1	\$27,150	\$ 4,052	\$ 31,202
2	27,150	4,052	31,202
3	27,150	4,052	31,202
4			

1003541021



## 1. Research Plan

A. Specific Aims

To evaluate the carcinogen enhancement of tumor growth as a method for the determination of carcinogens in complex mixtures such as those derived from air sampling. To relate these determinations to other measures of air pollution and carcinogenicity. To compare effects so detected in local areas with the cancer epidemiology of those areas. To isolate and identify the fractions in air samples that are capable of producing this effect.

B. Method of Procedure

The solid content of measured volumes of air will be collected in a standard manner with the assistance of the Houston Department of Health in consultation with the Robert A. Taft Center of the U.S. Public Health Service. Mobile collection equipment will be situated in places where good cancer frequency data already exists and where further detailed studies are being made by the Cancer Epidemiologists of the Texas Medical Center. These areas will include the environs of several urban areas together with comparatively rural sections where no abnormal pollution is present. These will be selected on the basis of location, population stability, and completeness of medical recording.

Chemical and analytical treatment of these samples will be patterned after the methods developed in cooperation with the Taft Sanitary Engineering Center. Similar types of fractions obtained from them will be compared with our materials.

Our present biological technique consists in the skin painting of DBA/2 mice with suspected carcinogens dissolved in benzene or other appropriate solvents. Groups of at least six young (2-4 month old) mice are painted (0.1 ml of mixture applied) three times a week for a total of six weeks. One week after the last painting a suspension of tumor cells (Gardner Lymphosarcoma - 6C3HED) is injected subcutaneously into the axillae of treated and untreated mice. The tumor is carried in our inbred line of C3H mice and is prepared in a standard manner designed not only to avoid the destruction of the whole cells but to separate them well. The usual dose of  $1$  to  $2 \times 10^7$  viable cells causes measurable growth by the fifth to sixth day. This either regresses within ten days or kills by progressive growth in 15-20 days.

In view of our being able to test small amounts of complex mixtures relatively rapidly, it is possible to further fractionate the mixtures beyond the present techniques of the Taft laboratory and thus to determine the active fractions. Identification of these fractions will be attempted with the help of such analytical facilities as infra red spectroscopy, mass spectroscopy, x-ray crystallography, and other methods available in nearby commercial laboratories.

Active fractions from different areas and situations will be compared and mixtures and fractions found effective in causing significant enhancement of tumor growth will be similarly painted on a group of 30 DBA/2 mice. This strain of mice has been repeatedly studied in this laboratory for spontaneous and carcinogenic development of neoplasms and the rates are well known and reproducible. These mice respond to known carcinogens within 18 months with greatly increased malignancy rates. Thus the induced cancer rate will be compared with controls and with the tumor enhancement phenomenon.

### C. Significance of this Research

Present methods of analysis for carcinogenic effect are slow. Moreover they require many animals and relatively large amounts of materials and time. The method we employ not only shows promise of correlating closely with classical methods of carcinogen detection. Thus we are in a position to fractionate complex mixtures found in air and put them to a sensitive test requiring only a small fraction of the test sample ordinarily needed. This could greatly speed up the identification of the offensive components of air pollution and perhaps help to identify the different carcinogens related to specific types of air pollution.

Although our assay is not one for carcinogenesis per se, this study which does compare it to classical methods may delineate the usefulness of this simple objective procedure.

The excellent cancer data collected in this area and state by the Texas Medical Center Epidemiology Services makes it possible to compare air studies to known clinical responses. In this way, carcinogenesis in mice as well as the tumor enhancing effect could be realistically evaluated in terms of actual human effects.

### D. Facilities Available

We have available a colony of about 25,000 mice of some 15 inbred strains. Our group of Db<sub>a</sub>/2 mice susceptible to the carcinogen enhancement effect is large enough to immediately initiate large scale studies.

We have complete biological and chemical laboratory facilities with all of the usual equipment. Also available for coordinated study is some of the air sampling equipment of the City of Houston and the use of excellent commercial analytical facilities.

## 2. Previous Work Done on this Project

We have been studying the occurrence of spontaneous and carcinogen induced neoplasms in several strains of mice (including the Db<sub>a</sub>/2) for about three years.

About two years ago, in connection with experiments concerning the effect of tumor immunity on the incidence of carcinogen-induced tumors, we noted that in Db<sub>a</sub>/2 mice (and related Db strains) the 6C3HED lymphosarcoma grew progressively after methylcholanthrene treatment.

Subsequently we showed that a variety of known carcinogens as well as x-ray had this effect. As little as 30 micrograms of 9,10-dimethylbenzanthracene on the skin of each mouse was sufficient to cause progressive growth in 50 per cent of treated mice. Much smaller quantities measurably increased the size of the tumors. Related hydrocarbons that are non-carcinogenic were without effect.

This effect was not related to the expression of immune response to

unrelated antigens (sheep or chicken erythrocytes) and was only very slightly affected by large doses of corticosteroids.

When mice susceptible to tumor growth enhancement with carcinogens are crossed with non-susceptible strains, all of the F1 progeny are non-susceptible. But 25% of F2's are able to respond. Thus we showed that this response was governed by a recessive genetic mechanism that is quite different from the invariably dominant H2 system.

The City of Houston engineers have been collecting air contaminants for more than three years and are well acquainted with related techniques.

### 3. Personal Publications

Rubin, B.A., N. Ida and A. Kirschbaum 1956 Growth enhancement of a transplanted mouse lymphosarcoma. Proc. Cancer Res. 2:143.

Rubin, B. A. and A. Kirschbaum 1956 The effect of antibiotics on the growth of a transplanted lymphosarcoma in mice. Bact Proc. 1956:66.

Rubin, B. A. and N. Ida Studies on the mechanism of growth enhancement of transplanted mouse tumors by carcinogens. Proc. Cancer Res. 2:244.

Rubin, B. A. and E. B. Ward 1957 A new genetic factor affecting tumors transplantation in mice. Genetics. in press.

### 4. Results Obtained by Others

The carcinogenic nature of air contaminants has been demonstrated by Clema, et al (1). Waller (2) has detected known carcinogens (3,4 benzpyrene) in numerous air samples. Much recent evidence showing the systemic effect of carcinogens is reviewed by Berenblum (3). The recent work of Baldwin (4) and of others indicates that carcinogens cause a decrease in the natural resistance of an animal to the growth of spontaneous tumors, which might otherwise be resisted. Other work by Malmgren, et al (5) and by others demonstrates further that changes in host susceptibility may be a significant facet of carcinogen activity.

(1) Clema, G.R., et al. Carcinogenic Action of City Smoke. Brit. J. Cancer. 9:137 (1955).

(2) Waller, R.E. The Benzpyrene Component of Town Air. Brit. J. Cancer. 6:8 (1952).

(3) Berenblum, I. Carcinogenesis and Tumor Pathogenesis, in Advances in Cancer Research. Vol. 2, Academic Press, New York, 1954.

(4) Baldwin, R.W. Immunity to Methylcholanthrene-Induced Tumors in Inbred Rats following Atrophy and Regression of Implanted Tumors. Brit. J. Cancer Res. 9:652 (1955).

(5) Malmgren, R.A., et al. Reduced Antibody Titers in Mice Treated with Carcinogenic and Cancer Chemotherapeutic Agents. Proc. Soc. Exptl. Biol. & Med. 79:484 (1952).

## 5. Biographical Sketches

Rubin, Benjamin Arnold. Ph.D. 1947, Yale University (Microbiology). Assistant Bacteriologist, U.S. War Department 1940-44; Research Microbiologist, Schenley Research Institute, 1944-45; Immunochemist, Yale University, 1945-47; Associate Microbiologist, Brookhaven National Laboratory, 1947-52; Chief Microbiologist, Syntex S.A., 1952-54; Research Associate, Department of Medicine, Baylor University College of Medicine, 1954; Asst. Professor of Preventive Medicine, Baylor University College of Medicine, 1955-present. Member.

REDACTED

REDACTED

REDACTED

Kemp, Hardy Alfred. M.D. 1926, St. Louis University. Interne, William Beaumont Gen. Hosp. El Paso, Texas, 1926-1927; Assoc. Prof. Bacteriology and Hygiene, Baylor University, 1928-34; Prof. Bacteriology and Preventive Medicine, 1934-39; Dean, College of Medicine, and Professor of Preventive Medicine, University of Vermont, 1939-41; Dean, College of Medicine, and Director of University Hospital, Ohio State University, 1941-44; On military leave, 1942-45; Dean, College of Medicine, and Professor of Preventive Medicine, Wayne University (Detroit), 1945-48; Professor of Preventive Medicine and Chairman, Dept. of Public Health and Preventive Medicine, Director of Graduate Studies, Baylor University College of Medicine, 1948-present; Div. Medical Consultant (Industry) Liberty Mutual Insurance Company, 1950-present.

Kirschbaum, Arthur. Ph.D. 1936; M.D. 1943, Minnesota. Alexander Brown Coxé Fellow, Finney-Howell Fellow, Instructor in Anatomy, Yale, 1937-41; Instructor, Assistant Professor, Associate Professor Anatomy, Minnesota, 1941-51; Professor and Head of Anatomy, Illinois, 1951-54; Professor and Chairman, Anatomy, Baylor University College of Medicine and University of Texas Dental Branch, Consultant M.D. Anderson Hospital and Tumor Institute, University of Texas, 1954-present. Societies:

REDACTED

REDACTED

Consultant, Public Health Service (Morphology and Genetics Study Section), 1951-present.

Love, Alan C. B.S. 1932, Texas A. & M (Sanitary Engineering); M.S. 1938, Harvard Univ., (Sanitary Engineering). Public Health Engineer, City of Houston, Reg. Professional Engineer; Austin-Travis County Health Unit, 1938-42; Public Health Engineer Waco-McClellan County Health Unit, 1942-45; Private Engineering Operations, 1945-51; Chief Engineer, City of Houston, Department of Health, 1951-present. Reserve Commission Public Health Service, 1941-45. Fellow Public Health Assn.;

REDACTED

REDACTED

Osdene, Thomas S. B.Sc. 1951 (1st class honors), University of London; Ph.D. 1955 (Organic chemistry), University of London. College Chemistry Prize, Birbeck College, University of London. Research Chemist, Chester Beatty Research Institute, London, 1951-55; Research Assistant, Princeton University, 1955-56; Assistant Biochemist, University of Texas, M. D. Anderson Hospital and Tumor Institute, September 1956-present. Societies:

REDACTED

TIRC Grant #181R1

Benjamin A. Rubin, Ph.D.  
Baylor University

CONFIDENTIAL

Report No. 2

November, 1959

An Evaluation of the Phenomenon of Tumor Growth  
Enhancement as an Assay for Carcinogens Among  
the Polycyclic Hydrocarbons and Related Compounds

The major part of our efforts during the past year in this program were related to the study of the mechanism and extent of carcinogen effect on transplantation; and on the quantitation of techniques for administration of chemicals and the measurement of growth effects.

Since we are unable, for reasons of time and money, to raise enough of our own DBA/2 and C3H mice, we have resorted to buying these strains from the Jackson Memorial Laboratory. This created a problem in re-standardizing the growth of our tumors and quantitating the effects of the carcinogens. Several of our old lines of the 6C3HED lymphosarcoma carried in CBA mice grow poorly (subcutaneously) in both C3H/JAX and carcinogen treated DBA/2 (Jackson) mice. There is a good correlation between the extent of growth in these two host types.

In the course of other experiments we isolated several new tumor lines from painted DBA/2 mice in which regressed tumors spontaneously reappeared. These "selected" tumors were then transplanted into a variety of other mice, including normal and treated DBA/2, C3H, and hybrids between these strains, and studied both as subcutaneous and as ascites tumors. While these studies are still in progress, we have already developed one tumor line that grows very well in carcinogen treated DBA/2 but hardly at all in normal mice of that strain. The growth in C3H/JAX is not quite as good as in the treated DBA/2 mice. Further work on the standardization of this tumor is in progress.

It may be pointed out, however, that the effect of the carcinogens on the growth of this tumor is very clear and very great. This was demonstrated in experiments where we measured the dose response at different levels of carcinogen treatment. We were able to show a large effect with a total skin application of  $1 \times 10^{-6}$  moles of material, equivalent to 25  $\mu$ g of methylcholanthrene. This was the smallest amount used in the test, but was far above the level of detectability. (see attached photograph)

This finding may be compared with our earlier result (in the USPHS Report submitted last year) in which the same amount of methylcholanthrene was barely at the level of detectability in the previous system of testing. Another innovation, besides the use of a new tumor line, is instead of presenting the carcinogen in a single dose (titration by varying the number of applications of the same solution), it is now given in 10 divided doses over a period of three weeks. In this system the number of applications remains

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constant, but the dilution of the test solutions is changed. The effect and significance of this technique will be discussed further in another context.

Another problem related to quantitation of the carcinogens concerns the question of chemical integrity. In discussions with Dr. Ralph Becker, he pointed out that some of the common polycyclic hydrocarbons were extremely labile. The question arises as to whether it is the pure hydrocarbon or its oxidation products that are carcinogenic. Becker feels that many substances of this type used in biological experiments are really only degradation products.

This possibility was tested here with 7,12 (9:10) dimethylbenzanthracene, one of the most easily oxidized compounds of our study. Solutions were prepared in spectroscopic grade benzene and then divided. Half was deoxygenated (with pure nitrogen) and kept in dark bottles at 5°C in the presence of polished iron wires. Samples of this were rapidly withdrawn for use and the residues never returned. The control (the other half of the same solutions) were kept in the usual dark bottles in the cold but without the other precautions. In tests lasting three weeks, the carefully handled materials had approximately twice the tumor growth enhancing effects of the control solutions.

It is therefore quite clear that our previous type of handling which is more careful than most biological testing methods described in the literature, is still quite inadequate for good quantitative comparison. The differences in biological effect can be greatly complicated (and perhaps obliterated) by the differences in chemical reactivity. It is also clear that in the case of this compound, at least, it is the original material and not the oxidation products that has the biological effect.

In the course of studies on the mechanism of action of polycyclic hydrocarbons it became evident that some substances could potentiate the ability of these compounds to enhance tumor growth. If this could be refined to a consistent effect, it might not only help elucidate the mechanism of carcinogenesis but would make the quantitative determinations more sensitive. This is especially valuable where the hydrocarbon supply is very small and cannot easily be replaced.

This phenomenon, of potentiating the carcinogen effect, was demonstrated in experiments using poorly growing tumors in DBA/2 mice that had been painted with different levels of methylcholanthrene. In this design, one could detect either a depression or an increase in the effect of the carcinogen.

The most striking effect was produced by Kinetin, and a smaller effect was also seen with Zymosan and Vitamin B12. All of these agents are thought to enhance immunological response. These observations were contrary to the concept held by some people that carcinogens depress the immune response. If this were true, antibody stimulating mechanisms should reverse carcinogen effect. In view of our results we must consider the possibility, suggested by H. N. Green and others, that carcinogens may

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actually stimulate an antibody response that can interfere with the animals normal immunity -- possibly by a mechanism similar to the "enhancement" described by Kaliss and others.

In this context the action of corticoids is difficult to interpret. While cortisone alone, given before the tumor has a growth enhancing effect, it has the opposite effect if it is presented after the tumor is already implanted. When given at the same time as the carcinogen, there was a marked synergistic effect on the growth of a tumor that failed to respond to the enhancing effect of the same dose of either agent alone. However, the characteristics of the tumor growth were different from that seen after carcinogen treatment alone (USPHS Report). The tumor tends to persist rather than grow and the animals show no development of immunity. Subsequent tumor implants can grow as well or better than the original tumors.

Another type of observation also supports the antibody stimulating effect concept. Different strains of mice were treated with carcinogens for various periods and then sacrificed. Their RE tissues, blood, marrow, kidneys, etc. were examined histologically. The most consistent finding was the early stimulatory effects on the lymph nodes and RE tissues in general. This was manifested by hyperplastic enlargement of the nodes and evidence of rapid proliferation of the lymphocytic elements.

It can now be pointed out again that the same amount of a carcinogen when given in divided doses over three weeks has more tumor enhancing effect than a single dose. This too might be interpreted as fitting the pattern of an immunological effect.

The continuation of our studies using the transplantation of skin as a criterion for the carcinogen effect has several advantages over the tumor studies. It is not possible, for instance, to test the effect of continued carcinogen treatment because of the interference with tumor growth. Similarly the effect of anti-metabolites which might interfere with antibody production also have anti-tumor effects. Progressive tumor growth also creates a maximum effect beyond which no further observation can be made. As we have previously reported, we can greatly improve the transplantability of homologous skin by carcinogen treatment. This effect was further improved by treating animals with Amethopterin after the foreign skin was already implanted. No improvement of effect was obtained by continuing treatment with the carcinogen after transplantation. Further experiments are in progress (based upon extension of the design, using antibody stimulating methods during pretreatment and suppressing agents after the skin is implanted) to determine what combination of treatments will extend the period of successful transplantation.

Another line of experimentation, (we reported this at the last Cancer Meeting) deals with the effects of carcinogen treatment on animals in parabiosis. Here too the findings support the idea that the carcinogen effect is producing a new characteristic in the animals, rather than just suppressing antibody response. These experiments were rather complex in concept, execution, and interpretation but the general conclusion could be summarized thus:

1) Antibody goes across the parabiotic barrier. This is indicated by the immunity of an animal that has itself had no previous tumor.

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2) A normal animal in parabiosis with a carcinogen treated one does not transfer its natural protective mechanisms to the susceptible (carcinogen painted) partner.

3) In the same combination, a normal animal becomes more susceptible to tumor growth, when in parabiosis with a treated mouse.

4) Tumor growth can be further stimulated in the normal animal by giving "enhancing" substances (in form of secondary tumor transplants) to the treated partners.

#### Summary

The work of the past year dealt with the standardization of tumor growth and response to carcinogens. The sensitivity of the tumor enhancing effect was increased both by use of new tumor-mice combinations as well as by alterations in technique. These changes arise from experiments pointing to a new interpretation of the effect in terms of increased immune (enhancing) response caused by the carcinogens. A new system of handling of carcinogens to avoid chemical change (oxidation) was developed, based on the finding that the usual precautions were inadequate and lead to a loss of biological effectiveness.

#### Plans for Future Studies

We are in the process of testing a variety of other tumor types and lines for their growth in carcinogen treated animals. Within the next three months or so we hope to have several stabilized systems with varying levels of sensitivity.

We also have in progress a large group of experiments using different substances that are shown to affect the carcinogen response. In one direction these findings may be used to further increase the quantitative response (as well as help elucidate the mechanisms involved). On the other hand, the possibility of interfering with carcinogens by a pharmacological method might have very great practical applications.

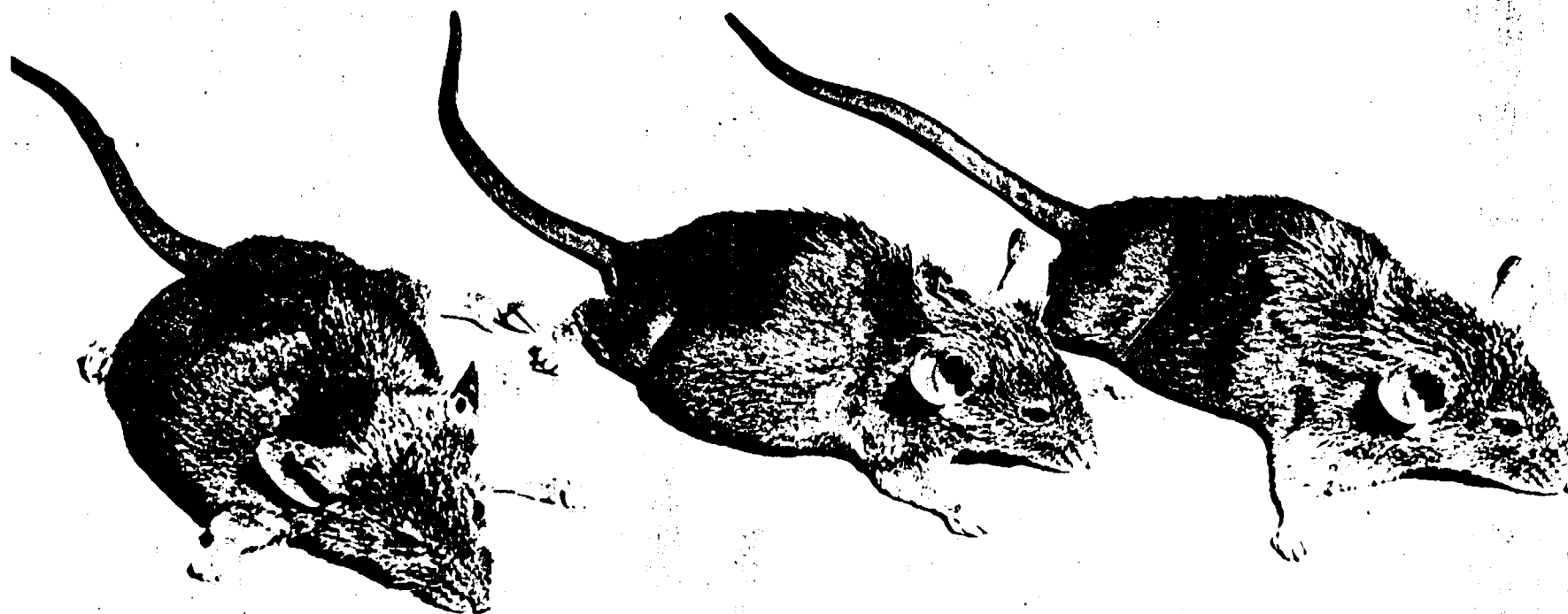
We are also working out detailed schedules of administration of the carcinogens to produce a predicable dose-response relation with agents of known effect. This is necessary before any quantitative comparison between different substances is really valid.

We hope that by mid-year most of these problems will have been resolved well enough to permit a large scale comparison of some of our more valuable analogous series of hydrocarbons.

We plan to work with Dr. Becker of the University of Houston in an attempt to correlate biological and chemical and properties; and also with Dr. Boutwell of University of Wisconsin to provide a comparison with a sensitive version of a classical test of carcinogenesis.

Experiments also continue on the effect of carcinogens on the transplantation of normal tissues. This is proving to be extremely promising from a theoretical as well as a practical viewpoint. Currently experiments are also underway on the homologous transplantation of bone marrow, as a further extension of the utility of these effects of the polycyclic hydrocarbons.

1003541029



TUMOR OF C3H ORIGIN GROWING IN DBA/2 MICE

Partly shaved  $\sigma$  DBA/2 mice 14 days after subcutaneous implantation of the 6C3HED Lymphosarcoma (on the right side). The mouse on the left had previously been treated with  $1 \times 10^{-5}$  moles of 3-methylcholanthrene, the center mouse had  $1 \times 10^{-6}$  moles by the same schedule. The animal on the right is an untreated control showing detectable tumor growth. For immobilization the mice had received 15 mg/kg of Thorazine.

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Committee:

Little, Chm.  
Jacobson  
Reimann

#181R2

(Originally activated 1/1/58;  
renewed - 1/1/59)

TOBACCO INDUSTRY RESEARCH COMMITTEE

150 East Forty Second Street

New York 17, N.Y.

Date: November, 1959

1. Name of Investigator: Benjamin A. Rubin
2. Title: Assistant Professor of Preventive Medicine
3. Institution & Address: Baylor University College of Medicine  
Texas Medical Center  
Houston, Texas
4. Project or Subject: An Evaluation of the Phenomenon of Tumor Growth Enhancement  
as an Assay for Carcinogens Among the Polycyclic Hydro-  
carbons and Related Compounds.
5. Detailed Plan of Procedure: -----
6. Budget Plan:

Salaries (G. B. Cobb, Technician)	\$ 3,710.00
Expendable Supplies (Chemicals, etc.)	2,000.00
Permanent Equipment	-----
Overhead (15%)	2,057.00
Other (Purchase and care of mice)	8,000.00
Total	\$15,767.00
7. Anticipated Duration of Work: 1 year. (Feb. 1, 1960-Jan. 31, 1961)
8. Facilities and Staff Available: -----
9. Additional Requirements: -----
10. Additional Information (Including relation of work to other projects and other  
sources of supply): -----

/s/ Dr. Stanley W. Olson  
Dean

/s/ Dr. Joseph L. Melnick  
Departmental Chairman

/s/ A. O. Johnson  
Business Manager

1003541031

TOBACCO INDUSTRY RESEARCH COMMITTEE  
150 EAST FORTY SECOND STREET NEW YORK 17, N. Y.

CF #50B  
Activated Feb. 1, 1955  
Renewed June 1, 1957

Application For Research Grant

Date: July 19, 1957

1. Name of Investigator: William O. Russell, M.D.
2. Title: Pathologist-in-Chief, M.D. Anderson Hospital and Tumor Institute;  
Professor of Pathology, University of Texas Postgraduate Medical  
School
3. Institution  
& Address: M.D. Anderson Hospital and Tumor Institute  
6723 Bartner Drive  
Houston 25, Texas
4. Project or Subject: Investigation, by whole organ subserial sections, of the possible  
carcinogenic effect of androgens and estrogens upon the respira-  
tory mucous membrane; correlation with studies of the respiratory  
tract of patients with bronchogenic carcinoma.
5. Detailed Plan of Procedure (Use reverse side if additional space is needed):  
(See Continuation sheets 1 to 5 for presentation of subject and  
Detailed Plan of Procedure.)

1003541032

### Introduction

The herein proposed investigations of the effects of hormones upon the human respiratory tract by whole organ subserial sections would be coordinated with a pulmonary cytology project currently being activated at the M.D. Anderson Hospital and Tumor Institute by the Field Investigations and Demonstrations Branch of the National Cancer Institute. The whole organ subserial technique to be utilized in the proposed correlative study was adapted for investigation of the human respiratory tract through the assistance of a grant from the Tobacco Industry Research Committee for anatomic-pathologic study of lungs.

One of the areas to be investigated in the pulmonary cytology project at this institution is the effect of sex hormones upon the cells in bronchial secretions of patients under treatment for cancer of the breast and prostate. Concomitantly, the pulmonary secretions of patients with lung cancer, as well as of patients from the clinic who have had a primary lung cancer removed, will be studied. The coordination of these two studies with each other and with studies of the pathologic changes in primary carcinoma of the lung and in the respiratory tract of patients who have received hormone therapy for carcinoma of the breast and prostate, by the method herein described, would provide an unique opportunity for the correlation of morbid anatomy with exfoliative cytology.

#### a) Specific Aim:

The study of the possible influence of sex hormones upon the rate of growth, replacement and morphology of the mucosal cells of the respiratory tract in relation to carcinogenesis.

#### b) Discussion of Problem:

One of the most significant advances in the field of cancer has been the recognition of the fact that the continued growth and development of certain types of neoplastic cells are influenced by sex hormones. In view of their proved beneficial effect upon cancer of the breast and of the prostate, the sex hormones have become established as a part of the treatment of these tumors.

It is not known whether the sex hormones produce any effect upon the cells of the respiratory tract. From the following facts, however, it would seem that the sex hormones might influence the rate of growth, replacement and morphology of mucous membrane cells and play some role in the development therein of neoplastic changes:

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(1) Adult male and female voices are characteristically different, the alteration in the male voice taking place at puberty. (2) The benign neoplastic conditions of juvenile papillomatosis of the larynx and juvenile nasal angiofibroma are restricted to the time of life when the sex hormones are assuming a new and heightened effect in the body. (3) Juvenile laryngeal papillomatosis has been reported to respond to treatment with sex hormones. (4) Following androgen therapy for breast cancer, gross changes in the laryngeal mucosa have been observed clinically. (5) The higher incidence of lung cancer in men than in women suggests that hormones may play some part in the pathogenesis of the disease.

Patients with metastatic breast cancer and almost all of those with prostate cancer at some time during the course of their disease receive large amounts of sex hormones, frequently in combination with ablation of hormone producing or stimulating tissues, such as ovaries, testes, adrenal glands and pituitary gland. As a result, the body tissues are subjected to a most abnormal hormonal change. These patients, therefore, would be excellent subjects for investigation and evaluation of the effects of hormones upon the cells of the human respiratory tract.

In the previously mentioned pulmonary cytology project at the M.D. Anderson Hospital and Tumor Institute, which is to be supported by the National Cancer Institute, bronchial secretions of patients with cancer of the breast and of the prostate who receive hormone therapy will be studied before, during and after treatment. In addition, control studies will be made of the secretions of individuals who do not receive hormones. Histopathologic examination of the respiratory mucous membranes of these subjects at autopsy would provide the ultimate opportunity for correlation of the morphologic changes with the cytologic findings in the bronchial secretions during hormone therapy. Although in most cases months or years would elapse between the hormone therapy and the necropsy, it may be assumed that careful studies, as herein planned, would permit the detection of any cellular morphologic changes which would be considered significant in the pathogenesis of lung cancer.

It is reasonable to assume a long latent period for bronchogenic carcinoma in relation to any exogenous and endogenous etiologic agents. If inhalation of ~~the~~ an exogenous substance, such as tobacco smoke, is a primary factor, and some hormone a co-factor, the period of latency from the first contact with the agents could be estimated as from 30 to 35 years. The fact that the disease reaches its peak incidence in persons around the age of 50 would substantiate this view. It has been shown that the latent period of pulmonary carcinoma arising from such known carcinogens as chromates and asbestos is much shorter. Relatively short periods of abnormal hormonal stimulation could reasonably incite potentially cancerous changes detectable by the herein contemplated study. These could explain many pulmonary cancers and possibly their high incidence in men.

#### c) Study Material:

During 1955 and 1956 the following cases were available for study at the M.D. Anderson Hospital and Tumor Institute:

1003541034

Site	New Cases	No Previous Surgery	Deaths	Autopsies
Prostate	64	42	8	7
Lung	124	107	27	23
Breast	<u>273</u>	<u>138</u>	<u>17</u>	<u>6</u>
<u>Totals</u>	463	287	52	36

During 1957 and 1958 the institution is expected to reach the limit of its clinical capacity; therefore, a material increase in the number of patients admitted for cancer of the breast, prostate and lung is anticipated. In addition, cases will be available through other clinical activities in the Texas Medical Center connected with the "cytology project." Within four years a sufficient number of cases can be collected to permit definite conclusions as to any pathologic changes in the respiratory tract of possible etiologic significance in pulmonary carcinoma.

The follow-up services of the Anderson Hospital Clinic can provide a larger number of autopsies than listed. Only a small percentage of patients die in the hospital, yet over 90 per cent are residents of Texas. With the cooperation of pathologists in other localities, lung specimens from those patients who have been under hormone therapy and cytologic observation, but have not died in this hospital, may be obtained from hospitals throughout the state. If no local pathologist is available in such cases, a member of the pathology staff of this institution would be sent to conduct the autopsy.

#### 5. Detailed Plan of Procedure:

A) Whole organ studies would be made of the larynx, trachea and bronchial tree of autopsy subjects following death from cancer of the breast and of the prostate, for which hormone therapy had been given.

B) For comparative purposes, lungs of patients who have died from bronchogenic carcinoma would be studied in like manner. The results reported by the Tobacco Industry pathology study group failed to show a striking association of such pathologic changes as squamous metaplasia or atypical hyperplasia with carcinoma; the proposed investigation, however, would be carried out in greater detail and thus would afford an opportunity for a more thorough and finite evaluation of this problem.

C) For control cases, the respiratory systems of autopsy subjects between the ages of 45 and 55 would be studied following death from diseases other than cancer. Cases of this type are readily available from the Medical Examiner's office in Houston.

D) All the case studies would include a carefully taken history with reference to tobacco smoking, for evaluation in the ultimate results.

1003541035



E) Subserial whole organ technique for study of the respiratory tract.-  
The procedure described below has been developed at the M.D. Anderson Hospital and Tumor Institute for studying the human respiratory tract at autopsy.

The larynx, trachea and lungs are removed en bloc and fixed in 10 per cent neutral formalin. The tracheobronchial tree is filled with formalin to insure the most rapid fixation of the mucosal tissues before total immersion. After seven to ten days of fixation in the formalin, the specimen is dissected and the sections are taken as follows:

The larynx is detached from the trachea and sectioned in the frontal plane. At approximately 3 cms. above the bifurcation, the trachea is cut transversely and divided into five or six rings for sectioning. The remaining trachea with the bifurcation and the two main stem bronchi are detached as a separate specimen for blocking. The detached lungs are decorticated by slicing off the excess parenchyma in layers with a knife, so as to preserve the major and minor bronchi as well as possible. Each lung specimen so prepared measures approximately 2.5 x 8 x 4 cms. (See Exhibit A, plate 1, showing specimens prepared for sectioning.)

The specimens are processed by the routine paraffin technique, each step being somewhat lengthened to accommodate the large tissue mass. After the tissues are embedded in paraffin, sections 8 microns in thickness are made on a Spencer sliding microtome at 1 mm. intervals and stained by hematoxylin and eosin, then mounted on glass slides of either 2 x 3 or 3 x 4 inch dimensions. By this method, an average of 35 slides are obtained from the larynx, and 10, 15 and 20 slides from each of the other specimens, respectively.

The linear surface of mucosa provided by the above described technique is, roughly, as follows:

	Average Number Sections	Cm. of Mucosa
Larynx	28	620
Trachea	44	900
Bifurcation and Primary Bronchi	15	167
Hilar Tissue and Principal Bronchi	(Rt. 22	5,000
	(Lt. 24	6,300
<u>Totals</u>	133	12,987

The above figures, showing an estimated 133 sections on each case, with an estimated 12,987 cms. of respiratory tract mucosal surface available for study, give some idea of the completeness of the examination and its usefulness in the detection and study of pathologic changes in the respiratory tract. (For pictorial evaluation of the whole organ sections, see Exhibit A, plates 2 and 3.)

1003541036

Budget Analysis:Budget Plan

Salaries . . . . .	\$14,000.00 <sup>004.<sup>00</sup></sup>
Expendable supplies . . .	2,000.00
Permanent equipment . . .	1,400.00
Overhead . . . . .	1,488.00
Travel . . . . .	<u>1,200.00</u>
<u>Total</u>	\$20,088.00 <sup>092.</sup>

a) Personnel:Itemized Salaries

Senior pathologist, part time . . . . .	\$ 6,000.00
Research technologist, II (histology) . . . . .	4,404.00
Senior clerk-typist, part time . . . . .	1,512.00
Research technologist (histology), half time . . . . .	1,824.00
Social Security Tax . . . . .	<u>264.00<sup>264.00</sup></u>
<u>Total</u>	\$14,000.00 <sup>004.<sup>00</sup></sup>

1) Senior pathologist, part time: A pathologist at an assistant or associate level would be necessary for the proper execution of this project. Thorough qualification in pathologic anatomy would be a prerequisite, since the study and interpretation of many thousands of sections would be the major professional assignment of the project. It is estimated that one-third or more of the time of such a person would be devoted to this work.

2) Research technologist, II (histology): The entire program would be dependent upon the preparation of whole organ sections, for which specialized training and skill in the various techniques is essential on the part of the medical technologist.

3) Senior clerk typist, part time: Typing and clerical assistance would be necessary for obtaining data on cases to be studied, for preparation of forms, correspondence regarding outside information on patients and autopsy studies, and for preparation of reports and papers for publication.

1003541037

4) Research technologist (histology), half time: It is estimated that half the time of a superior tissue technologist with some experience in whole organ section technique would be necessary. This person would work with and under the Research Technologist, II.

b) Expendable supplies.- In the preparation of whole organ sections, supplies are consumed at a rapid rate. The slides and cover slips are custom cut. Reagents are used in large quantities, and considerable numbers of staining receptacles, miscellaneous glassware and bottles are required.

c) Permanent equipment.- Two low power scanning microscopes of the Zeiss cytoplast type would be needed. The pathologist would utilize one in scanning the sections, and the technologists would use one in staining procedures for evaluation of intensity of stains and general excellence of preparation. The laboratory has all other permanent equipment necessary to produce whole organ sections and as outlined.

d) Travel.- Travel funds would be used by persons engaged in this program for the purpose of consulting with others regarding problems related to the project and to report results of studies at scientific meetings. It would also be necessary for staff members of the Department of Pathology to travel to other parts of Texas to perform autopsies on selected patients following death in their local hospitals. To illustrate the expense involved in intrastate travel in Texas, the cost of a round trip plane passage between Houston and El Paso is \$96.25.

It is anticipated that the consumable supply and travel expenses will increase after the first year as the program becomes fully activated. This will be offset by a reduction in permanent equipment expense after the first year.

#### 10. Relationship of Proposed Study to Pulmonary Cytology Project:

A cooperative research program between the M.D. Anderson Hospital and Tumor Institute and the Field Investigations and Demonstrations Branch of the National Cancer Institute is being activated in Houston for the study of pulmonary cytology. The objectives of the project are as follows:

- a) Cytologic examination of sputum and/or bronchial washings to determine general smear characteristics and specific cellular morphology.
- b) Development and investigation of improved methods for collection of sputum specimens for cytologic study. This will include both chemical and mechanical methods.
- c) Development and investigation of methods of preservation, processing and staining of sputum and/or bronchial washings.
- d) Evaluation of screening methods for examination of prepared specimens.

One of the initial projects will be a study of the effects of hormone therapy, given to patients for mammary and prostatic cancer, upon the cells in secretions from their respiratory tracts, as well as cellular changes in secretions from the respiratory tracts of normal subjects. This field of exfoliative cytology is untouched. Such a study will provide an unusually complete background for the whole organ investigations of the respiratory system of cancer subjects included in the *proposed project*

1003541038

6. Budget Plan:

Salaries	<u>\$14,865.00</u>
Expendable Supplies	<u>2,000.00</u>
Permanent Equipment	<u>1,400.00</u>
Overhead 7.1%	<u>1,488.00</u>
Other	<u>1,200.00</u>
Total	<u>\$20,953.00</u>
	092.

(See Continuation sheets 6 and 7.)

7. Anticipated Duration of Work:

Four years will be needed to collect a sufficient number of cases to be of significance in the evaluation of this problem.

8. Facilities and Staff Available:

New, completely equipped laboratories for anatomic and clinical pathology are available in a 310 bed center for cancer education, research and treatment. The staff of the Department of Pathology consists of six senior pathologists and seven fellows. For the past seven years the Department of Pathology has been working on the development of whole organ subserial paraffin sections. It will be possible to obtain qualified technologists through the training program for histologic technologists at the M.D. Anderson Hospital and Tumor Institute.

9. Additional Requirements:

None.

10. Additional Information (Including relation of work to other projects and other sources of supply):

(See Continuation sheet 8.)

Signature /s/ William O. Russell  
Director of Project

/s/ Joe E. Boyd, Jr.  
Business Officer of the Institution

1003541039

Application For Research Grant

Originally activated on  
February 1, 1955

Date:

June 1st, 1957

1. Name of Investigator:

William O. Russell, M.D.

2. Title:

Pathologist-in-Chief

3. Institution  
& Address:

University of Texas M. D. Anderson Hospital and  
Tumor Institute,  
6723 Bertner Drive, Houston 25, Texas.

4. Project or Subject:

Pathologic-Anatomic Study of Cellular Changes in  
Human Lungs.

These investigations are part of the group study initiated for the Tobacco Industry Research Committee by Dr. Stanley P. Reisman. They will be concerned with the continued pathologic examination of the tracheobronchial tree by a subserial whole organ technique to ascertain changes occurring there as a result of inhalation of exogenous substances, as may be indicated in this group research program.

5. Detailed Plan of Procedure

(Use reverse side if additional space is needed):

The subserial whole organ technique of examination of the larynx, trachea and tracheobronchial tree has been successfully adapted and demonstrated to be of practical significance for the pathologic-anatomic study of cellular changes in human lungs. Results to date have been most revealing as to the opportunity afforded for examination of, not only the mucosal lining of the entire respiratory tract from the larynx to the tertiary bronchioles, but also, the anatomic relationship to the mucosa of all adjacent tissues.

1003541040

6. Budget Plan:

Salaries	2,106.00
Expendable Supplies	892.00
Permanent Equipment	47.00
Overhead	243.00
Other	(85)
Social Security	
Total	3,298.00

7. Anticipated Duration of Work: **Six months**

8. Facilities and Staff Available: **Adequate facilities and staff available.**

9. Additional Requirements: **None.**

10. Additional Information (Including relation of work to other projects and other sources of supply):

1003541041

Signature \_\_\_\_\_  
Director, of **William O. Russell**

Business Officer of the Institution  
**W. S. Boyd Jr.**

3

1003541042



Source: <https://www.industrydocuments.ucsf.edu/docs/mnpl0000>

9-13 1955

From the desk of:

ROBERT N. DU PUIS

To:

~~AEON~~  
~~GUM~~  
~~RTS~~  
~~JG~~  
~~FR~~  
JCS

Return by Oct. 10

RD

How about smoking some of these, containing specifically labeled organic compounds and analyze the products using gas chromatography; the detecting device could be a Geiger counter and mass spectrum would provide positive identification. RBS

We need a heated inlet system to run these compounds in the mass spectrometer.

A quantitative measure of the amount of  $\text{CO}_2$  exchanging with the leaves could be easily determined with the mass spectrometer. J.E.R.

1003541044

HPH  
August 16, 1955

Dr. Paul Kotin  
School of Medicine  
1200 N. State Street  
Los Angeles 33, California

Dear Dr. Kotin:

In our conversation the other day you were interested in the significance of the unique labeling encountered during the dark fixation of  $C^{14}O_2$  by tobacco leaves. It should be emphasized that only the organic acid fraction and those few amino acids which are formed by transamination from  $\alpha$ -keto acids will become radioactive even after extended periods of time. None of the carbohydrates or phosphorylated compounds pick up the label. If the alkaloids are labeled, it is at such a low level that we are unable to detect it.

Thus a method is available for the selective labeling of compounds within a tobacco leaf. If one were interested in the fate of the organic acids during the course of combustion of the cured leaf, it would be a simple matter to expose leaves to  $C^{14}O_2$  in the dark then transfer them to the curing shed without exposure to light. It should be emphasized that in the presence of light the fixed  $CO_2$  will be metabolized to all of the products normally encountered in photosynthesis.

If there is any further information that would interest you, please do not hesitate to call or write.

Sincerely,

Paul Saltman, Ph.D.  
Assistant Professor

1003541045

Application to the Tobacco Industries' Research Committee

for renewal of their support of a Project entitled

THE ENZYMATIC MECHANISM FOR THE DARK FIXATION OF CO<sub>2</sub> BY TOBACCO

from

Department of Biochemistry and Nutrition

University of Southern California

\* \* \*

Responsible Investigator:

Paul D. Saltman, Ph.D.  
Assistant Professor

Institution:

University of Southern California  
School of Medicine  
Department of Biochemistry and Nutrition  
Los Angeles 7, California

Scientific Personnel:

Paul D. Saltman, Ph.D.  
Clyde Stitt, B.S., (Research Assistant)  
Herbert Spolter, B.S., (Pre-doctoral Fellow)

Duration:

One year

Amount of Grant:

\$7,776.00

1003541046

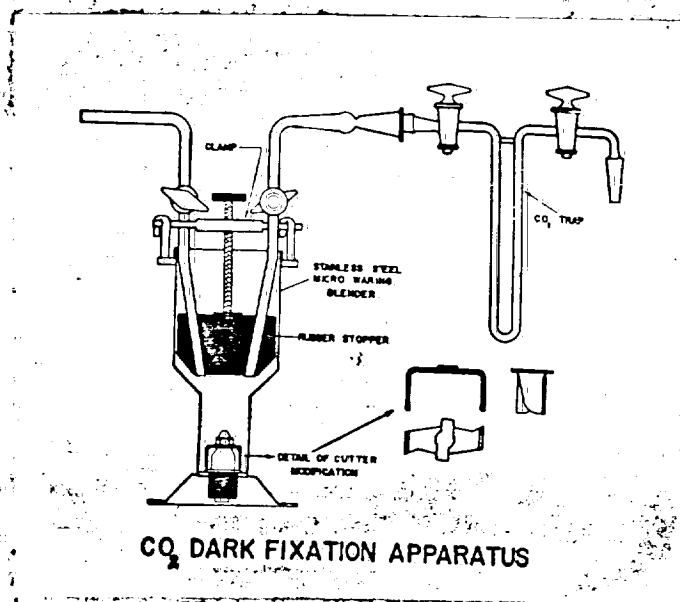
FOR THE TOBACCO INDUSTRIES' RESEARCH COMMITTEE:

Annual Report and Renewal of Contract

\*\*\*\*\*

THE YEAR'S PROGRESS: Our problem was to investigate the metabolic processes in tobacco leaves that are operative in the dark fixation of  $\text{CO}_2$ . Our approach was to identify the first products of the dark fixation by means of paper chromatography. Once these products were known, we planned to proceed with the isolation and study of the enzymatic system that mediates the inter-conversion of the products of the fixation.

An apparatus was designed that would permit the tobacco leaves to be exposed to high concentrations of  $\text{C}^{14}\text{O}_2$  in the dark and, after short periods of exposure to the radioactive material, to be homogenized with 80% ethyl alcohol to stop the reaction. A schematic drawing of the equipment is shown in Figure 1.



1003541047

It consists of a stainless steel micro-Waring Blender top with slightly modified blades to permit total homogenization of small leaves. The head is fitted with a two-hole stopper machined to give very close fit. The stopper with appropriate ground glass joints is held in position with the clamp as shown. All glass parts are coated with an opaque paint in order to exclude all light.

About one gram of young tobacco leaves (footnote 1) are placed around the blade of the blender to insure rapid and thorough homogenization. The blender is evacuated and the  $\text{CO}_2$ , previously generated and collected in a trap, is admitted into the closed system.

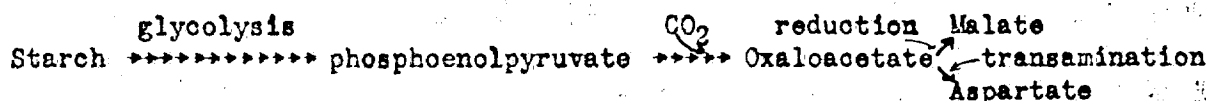
At the end of the exposure period, 30 ml. of boiling 80% ethanol is drawn into the reaction chamber by means of an aspirator and the leaves simultaneously homogenized. Exposure periods as short as one minute have resulted in the successful fixation of  $\text{CO}_2$  using the above procedure.

The alcoholic extract is centrifuged and stripped under vacuum to a final volume of 2.0 ml. One-tenth of a ml. of the concentrated leaf extract is placed on a large sheet of Whatman No. 1 filter paper along with authentic samples of suspected intermediates. The compounds are resolved by two-dimensional paper chromatography using the solvents 80% phenol - 20% water and butanol - acetic acid - water mixtures. The dried chromatograms are placed in contact with no screen X-ray film to locate the spots that have incorporated the radioactivity. The chromatograms are then sprayed with brom cresol green to locate the organic acids and then sprayed with ninhydrin to locate the amino acids.

(Notes:) Seeds of Dixie Bright Tobacco were obtained through the generosity of Dr. Guy Jones, University of North Carolina. Plants were grown by Dr. F. Went at the California Institute of Technology.

1003541048

During a one-minute exposure of tobacco to  $C^{14}O_2$ , only two compounds incorporate the label: malic acid and aspartic acid. Significantly, this is the same pattern observed in parallel experiments with succulent leaves. This evidence suggests the following mechanism for the initial fixation:



Attempts to isolate oxaloacetic acid from tobacco as well as from other leaves have been unsuccessful. Communications with Dr. Aranoff and Dr. Thompson who have been concerned with similar experiments, reveal that it is a most difficult if not impossible compound to identify in green leaves. This does not, however, preclude its participation in the reaction above. As another approach to the problem, we have prepared and examined the acetone powders of tobacco leaves for the enzyme mediating the initial fixation, phosphoenolpyruvyl-carboxylase. This enzyme is present in quite high concentration in this tissue.

Exposure of the leaves to  $C^{14}O_2$  for periods as long as two hours causes the incorporation of the label into the following compounds: citric, isocitric, malic, fumaric, succinic acids, as well as alanine, aspartic acid, glutamic acid and glutamine. After five minutes exposure, all of the above compounds incorporate the label. These results are most interesting in the light of the similar patterns observed in succulent leaves. The succulents, however, incorporate the major fraction of activity into malic acid, whereas in tobacco leaves, the major fraction seems to be in aspartic acid.

1003541049



Dark exposed leaves have been allowed to accumulate  $C^{14}O_2$ , then removed to a light chamber and permitted to photosynthesize. At various intervals of time the photosynthesizing leaves were homogenized and extracted and the compounds identified as indicated above. The most significant observation was that the label disappears from the amino acid fraction quite rapidly but is not incorporated into the sugar fraction as one would expect.

PROPOSAL FOR FUTURE RESEARCH: Our interests are now directed toward the elucidation of the mechanisms operative in the light which cause the loss of radioactivity from the labeled amino acids. It is significant that in the tobacco leaves the major storage fraction for fixed  $CO_2$  in the dark is the amino acid, aspartic acid. Why? Is the transamination reaction from oxalo-acetic acid to aspartic acid of such magnitude to drive the dark fixation toward the amino acid rather than toward the Krebs cycle as found in succulents?

We are concerned that the  $CO_2$  fixed in the dark does not enter into the reversal of glycolysis to yield radio-labeled sugar. It is of interest to understand what is the biochemical role of the fixed carbon dioxide in these green leaves. We would be interested also in determining the effects of various light intensities on the rate of transformation of the amino acids. Is there a key enzyme system which is light sensitive? Or is the phenomenon, rather, the result of a general increase in reducing power within the leaf.

1003541050

BUDGET:	Salaries:	\$5,000.00
	Expendable supplies:	1,200.00
	Permanent equipment:	1,000.00
	Overhead (8%):	<u>576.00</u>
TOTAL:		\$7,776.00

ANTICIPATED DURATION OF WORK: We feel that the major goals of the research program can be attained within the next year. Now that our laboratory is at its full working capacity and the new equipment has been developed and tested, we are certain that even more rapid progress may be made in the future.

FACILITIES AND STAFF AVAILABLE: Through the generosity of the Tobacco Industries Research Committee, our laboratory has now acquired equipment and materials which greatly facilitate our work with radioactive traces. The cooperation of Dr. Fritz Went of the Earhart Plant Research Laboratory at the California Institute of Technology has been placed at our disposal and he has provided us the only means in southern California for the growing of smog-free tobacco leaves.

Our staff has been deprived of the services of Dr. Vicke Haas Lynch who has taken a position at the Carnegie Institute of Biological Research. However, before her departure, she trained Mr. Stitt in the complexities of the art and science of chromatography. Mr. Stitt has become a most proficient and able worker in this laboratory.

Mr. Herbert Stolter is a first year graduate student with a fine academic record both at the University of Southern California and at New York University. We hope that he will be able to make significant contri-

1003541051

butions through his research.

ADDITIONAL REQUIREMENTS: We are in need of a paper electrophoresis apparatus in order to identify the several unknown compounds appearing on the chromatograms. We are also in need of funds to aid in the purchase of a Beckman DU spectrophotometer to replace the one that has been removed from our laboratory by its original owner. Our group plans to combine its financial resources with two other groups of investigators in the bio-chemistry department to purchase another instrument.

ADDITIONAL INFORMATION: We have been pleased with the response of the students at this university to our increased emphasis in plant bio-chemistry. We hope to intensify this interest and to encourage more students to enter the field. In line with such a program, we have invited Dr. Bernard Axelrod of Purdue University to be a guest lecturer in the bio-chemistry department in the summer of 1956. Dr. Axelrod is one of the foremost authorities in the field of plant bio-chemistry and will give a course in this field. He has also agreed while on this campus to actively participate in the program of  $\text{CO}_2$  fixation.

1003541052

TIRC - Res. Grants  
Saltman

# PHILIP MORRIS & CO. LTD., INC.

*Dedicated to the Production of Fine Tobacco Products*

NEW YORK, N. Y. · LONDON, ENGLAND

RICHMOND, VA. · LOUISVILLE, KY.

CABLE ADDRESS



"POLD NEW YORK"

P. O. BOX 1895  
**RICHMOND-15, VA.**

*Returned.  
File letter.*

August 25, 1955 *Repts. to be ret'd. to  
Kotin*

Mr. H. R. Hanmer  
American Tobacco Co., Inc.  
Research Laboratory  
400 Petersburg Pike  
Richmond, Virginia

Dear Rupert:

At the Scientific Advisory Board meeting this week, Paul Kotin showed me the two attached items from Dr. Paul Saltman with reference to his work under TIRC grant on the dark fixation of carbon dioxide by tobacco. In view of your interest and experience in this field, I told him that I was sure you would be interested in reading these communications. After you have perused them, I would appreciate their return. Although Dr. Kotin did not mention this, I presume you would do well to consider them confidential until they are published.

Best regards.

Sincerely,

*RD*  
Robert N. DuPuis  
Vice President - Research

RND:BS  
Enc.

1003541053

NRH ✓  
WRH ✓  
ESH ✓

RECEIVED  
FEDERAL BUREAU OF INVESTIGATION  
U. S. DEPARTMENT OF JUSTICE

FROM NEW YORK

TO BUREAU

RECEIVED  
RICHMOND-127A

*Handwritten:*  
L. J. ...  
J. ...

*Handwritten:*  
August 23, 1955

Mr. H. R. ...  
American Tobacco Co., Inc.  
Research Laboratory  
400 Patterson Pike  
Richmond, Virginia

Dear Sir:

At the Scientific Advisory Board meeting this week, Paul Rothman and the two guests from Dr. Paul Saltman with reference to his work with the group on the mechanism of action of tobacco by tobacco. In view of Paul's interest and experience in this field, I told him that I was sure you would be interested in reading these communications. After you have perused them, I would appreciate your return. Although Dr. Rothman did not mention this, I presume you would be well to consider them confidential until they are published.

Very truly,  
Sincerely,  
L. J. ...

Mr. H. R. ...  
American Tobacco Co., Inc.

END:33  
ENC.

1003541054

*Received*  
8/26/55

*quite basic  
Lawson?*

**Application to the Tobacco Industries' Research Committee**

**for renewal of their support of a Project entitled**

**THE ENZYMATIC MECHANISM FOR THE DARK FIXATION OF CO<sub>2</sub> BY TOBACCO**

**from**

**Department of Biochemistry and Nutrition**

**University of Southern California**

\* \* \*

**Responsible Investigator:**

**Paul D. Saltman, Ph.D.  
Assistant Professor**

**Institutions:**

**University of Southern California  
School of Medicine  
Department of Biochemistry and Nutrition  
Los Angeles 7, California**

**Scientific Personnel:**

**Paul D. Saltman, Ph.D.  
Clyde Stitt, B.S., (Research Assistant)  
Herbert Spolter, B.S. (Pre-doctoral  
Fellow)**

**Duration:**

**One Year**

**Amount of Grant:**

**\$7,776.00**

1003541055

FOR THE TOBACCO INDUSTRIES' RESEARCH COMMITTEE;

Annual Report and Renewal of Contract

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1003541056

It consists of a stainless steel micro-Waring Blender top with slightly modified blades to permit total homogenization of small leaves. The head is fitted with a two-hole stopper machined to give very close fit. The stopper with appropriate ground glass joints is held in position with the clamp as shown. All glass parts are coated with an opaque paint in order to exclude all light.

About one gram of young tobacco leaves (footnote 1) are placed around the blade of the blender to insure rapid and thorough homogenization. The blender is evacuated and the  $\text{CO}_2$ , previously generated and collected in a trap, is admitted into the closed system.

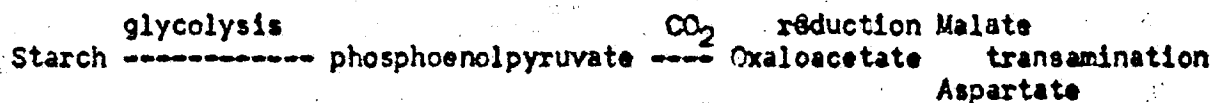
At the end of the exposure period, 30 ml. of boiling 80% ethanol is drawn into the reaction chamber by means of an aspirator and the leaves simultaneously homogenized. Exposure periods as short as one minute have resulted in the successful fixation of  $\text{CO}_2$  using the above procedure.

The alcoholic extract is centrifuged and stripped under vacuum to a final volume of a 2.0 ml. One-tenth of a ml. of the concentrated leaf extract is placed on a large sheet of Whatman No. 1 filter paper along with authentic samples of suspected intermediates. The compounds are resolved by two-dimensional paper chromatography using the solvents 80% phenol - 20% water and butanol - acetic acid - water mixtures. The dried chromatograms are placed in contact with no screen X-ray film to locate the spots that have incorporated the radioactivity. The chromatograms are then sprayed with brom cresol green to locate the organic acids and then sprayed with ninhydrin to locate the amino acids.

(Notes) Seeds of Dixie Bright Tobacco were obtained through the generosity of Dr. Guy Jones, University of North Carolina. Plants were grown by Dr. F. Went at the California Institute of Technology



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1003541058

Dark exposed leaves have been allowed to accumulate  $\text{C}^{14}\text{O}_2$ , then removed to a light chamber and permitted to photosynthesize. At various intervals of time the photosynthesizing leaves were homogenized and extracted and the compounds identified as indicated above. The most significant observation was that the label disappears from the amino acid fraction quite rapidly but is not incorporated into the sugar fraction as one would expect.

PROPOSAL FOR FUTURE RESEARCH Our interests are now directed toward the elucidation of the mechanisms operative in the light which cause the loss of radioactivity from the labeled amino acids. It is significant that in the tobacco leaves the major storage fraction for fixed  $\text{CO}_2$  in the dark is the amino acid, aspartic acid. Why? Is the transamination reaction from oxalo-acetic acid to aspartic acid of such magnitude to drive the dark fixation toward the amino acid rather than toward the Krebs cycle as found in succulents?

We are concerned that the  $\text{CO}_2$  fixed in the dark does not enter into the reversal of glycolysis to yield radio-labeled sugar. It is of interest to understand what is the biochemical role of the fixed carbon dioxide in these green leaves. We would be interested also in determining the effects of various light intensities on the rate of transformation of the amino acids. Is there a key enzyme system which is light sensitive? Or is the phenomenon, rather, the result of a general increase in reducing power within the leaf.

1003541059

BUDGET:	Salaries:	\$5,000.00
	Expendable supplies:	1,200.00
	Permanent equipments:	1,000.00
	Overhead (8%):	<u>576.00</u>
	TOTAL	\$7,776.00

ANTICIPATED DURATION OF WORK: We feel that the major goals of the research program can be attained within the next year. Now that our laboratory is at its full working capacity and the new equipment has been developed and tested, we are certain that even more rapid progress may be made in the future.

FACILITIES AND STAFF AVAILABLE: Through the generosity of the Tobacco Industries Research Committee, our laboratory has now acquired equipment and materials which greatly facilitate our work with radioactive tracers. The cooperation of Dr. Rik Fritz Went of the Earhart Plant Research Laboratory at the California Institute of Technology has been placed at our disposal and he has provided us the only means in southern California for the growing of smog-free tobacco leaves.

Our staff has been deprived of the services of Dr. Vickie Haas Lynch who has taken a position at the Carnegie Institute of Biological Research. However, before her departure, she trained Mr. Stitt in the complexities of the art and science of chromatography. Mr. Stitt has become a most proficient and able worker in this laboratory.

Mr. Herbert Stolter is a first year graduate student with a fine academic record both at the University of Southern California and at New York University. We hope that he will be able to make significant contri-

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butions through his research.

ADDITIONAL REQUIREMENTS: We are in need of a paper electrophoresis apparatus in order to identify the several unknown compounds appearing on the chromatograms. We are also in need of funds to aid the purchase of a Beckman DU spectrophotometer to replace the one that has been removed from our laboratory by its original owner. Our group plans to combine its financial resources with two other groups of investigators in the bio-chemistry department to purchase another instrument.

ADDITIONAL INFORMATION: We have been pleased with the ~~xm~~ response of the students at this university to our increased emphasis in plant bio-chem-~~istry~~ istry. We hope to intensify this interest and to encourage more students to enter the field. In line with such a program, we have invited Dr. Bernard <sup>X</sup>Axelrod of Purdue University to be a guest lecturer in the bio-chemistry department in the summer of 1956. Dr. Axelrod is one of the foremost authorities in the field of plant bio-chemistry and will give a course in this field. He has also agreed while on this campus to actively participate in the program of CO<sub>2</sub> fixation.

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ADMINISTRATIVE APPROVAL

This proposal is approved by

The University of Southern California  
School of Medicine  
Department of Biochemistry and Nutrition

s/ Paul D. Saltman

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Paul D. Saltman  
Responsible Investigator

s/ John W. Mehl, Head

Dr John W. Mehl, Head  
Department of Biochemistry  
and Nutrition

University of Southern California

s/ Fred D. Fagg  
Fred D. Fagg  
President

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TOBACCO INDUSTRY RESEARCH COMMITTEE  
150 EAST FORTY SECOND STREET

NEW YORK 17, N.Y.

#142 R 1

Activated 1/1/57  
Cf #6 activated  
on 10/1/54 and  
renewed 10/1/55

Renewal of Research Grant

Application ~~Renewal of Research Grant~~

Date: October 14, 1957

1. Name of Investigator: Paul D. Saltman, Ph.D.
2. Title: Assistant Professor  
Department of Biochemistry and Nutrition
3. Institution  
& Address: University of Southern California  
Los Angeles 7, California
4. Project or Subject:

Some aspects of amino acid metabolism in tobacco leaves. That several amino acids incorporate  $C^{14}O_2$  in the dark has been demonstrated in this laboratory using tobacco leaves. The biochemical pathways which are operative in the biosynthesis of these amino acids is at present unknown. It is proposed to investigate the enzymatic pathways mediating the metabolism of some of these important compounds.

5. Detailed Plan of Procedure (Use reverse side if additional space is needed):

During our first year of this grant we have been able to show conclusively that all of the serine produced during the dark fixation of  $CO_2$  by tobacco leaves is in the carboxyl carbon. Absolutely no radioactive carbon is to be found in the alpha or beta positions. This would seem to indicate a direct transformation from some three carbon precursor at the level of an organic acid. We have made extensive searches on our chromatograms to ascertain if any phosphorylated intermediate does indeed incorporate  $CO_2$  which would be on the pathway leading to serine. None was to be found except for phosphoglyceric acid. Radioactive pyruvate is also present and this could conceivably also lead into a three carbon precursor for serine.

Our attack now has shifted to the isolation of the enzymes involved in these reactions. It seems likely on the basis of the chromatographic data that the pools of these compounds present in the leaf are much too small to be measured. We therefore intend to work with homogenates with the hope that we can then isolate some of the intermediates in the pathway leading to serine biosynthesis.

We have also begun some work concerning the mechanisms of protein synthesis in tobacco and other plant as well as animal tissue. This work is now focused at the level of learning of the mechanisms involved in the activation of amino acids prior to their incorporation into the protein fraction of the leaf or cell. Mrs. Esther Allen is now actively engaged in this project and there seems to be a great promise for future developments along this line. Although this work was not in our original proposal, we felt it was of sufficient interest to warrant our investigations in this field.

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6. Budget Plan:

Salaries	\$5,000
Expendable Supplies	1,000
Permanent Equipment	1,000
Overhead	560
Other	
Total	\$7,560

7. Anticipated Duration of Work: 1 year

8. Facilities and Staff Available:

We have secured the cooperation of Dr. Fritz Went at California Institute of Technology who has placed the facilities of the Earhart Laboratories at our disposal for growing plants. We have a well equipped laboratory for biochemical investigations including: centrifuges, radioactive counting equipment, chromatographic equipment, cold rooms, spectrophotometer, etc.

Staff: Paul Saltman, Ph.D.  
 Esther Allen (pre-doctoral fellow)  
 Clyde Stitt (research assistant)

9. Additional Requirements:

10. Additional Information (Including relation of work to other projects and other sources of supply):

We have already published one paper concerning the work supported by the Tobacco Industries Research Committee concerning the metabolic pathways of the dark fixation carbon dioxide. We have also presented papers concerning further research along these lines at the last meeting of the Plant Physiological Society. The manuscripts for these papers are now in preparation and will be submitted to leading biochemical and plant physiological journals shortly.

Signature Paul Saltman  
 Director of Project

A.V. Call  
 Business Officer of the Institution  
 President of the Board

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TIRC Grants  
# 6 & 142

## DARK FIXATION OF CO<sub>2</sub> BY TOBACCO LEAVES<sup>1,2,3</sup>

GEORGE KUNITAKE, CLYDE STITT AND PAUL SALTMAN

DEPARTMENT OF BIOCHEMISTRY AND NUTRITION, SCHOOL OF MEDICINE, UNIVERSITY OF SOUTHERN CALIFORNIA,  
LOS ANGELES 7, CALIFORNIA

The ability of non-succulent plants to fix CO<sub>2</sub> in the absence of light has been previously demonstrated by several investigators (2, 5, 11, 17, 21). However, the succulents have received the greatest attention because of the large amounts of CO<sub>2</sub> incorporated into organic acids in the dark.

Although these earlier reports strongly suggested that dark fixation of CO<sub>2</sub> is an ubiquitous phenomenon in plants other than succulents, the nature of the biochemical reactions involved in this process has not been critically examined. Concurrently with the studies carried out in our laboratory on succulent metabolism (12, 13) we have investigated non-succulent dark CO<sub>2</sub> metabolism in *Nicotiana tabacum*. Studies concerning the nature of the initial carboxylation reaction and the fate of the fixed C<sup>14</sup>O<sub>2</sub> during the subsequent metabolic reactions in the absence of light were carried out with excised tobacco leaves. These studies show that the pathways for the dark metabolism of CO<sub>2</sub> by succulent and non-succulent leaves appear to be fundamentally the same.

### MATERIALS AND METHODS

The methods used in the study of the dark fixation of CO<sub>2</sub> in excised tobacco leaves are the same as those described in detail in earlier communications (12). *Nicotiana tabacum* (var. Hicks) plants used in these experiments were grown at the Earhart Plant Research Laboratory, Division of Biology, California Institute of Technology, Pasadena, California and most generously supplied by Dr. H. R. Highkin. Immediately before use, approximately 1 g of young leaves (5 to 7 cm long) were taken from the apex of the plants. The leaves were placed in an apparatus which permits exposure to C<sup>14</sup>O<sub>2</sub> in total darkness. After equilibration of the leaves for 5 minutes in the dark to remove any transient reducing compounds of photosynthesis, C<sup>14</sup>O<sub>2</sub> generated from 5.0 mg BaC<sup>14</sup>O<sub>3</sub> (specific activity 120  $\mu$ c/mg) was admitted into the chamber. After suitable exposure to C<sup>14</sup>O<sub>2</sub>, the reaction was terminated by homogenization in boiling 80 % ethanol.

The ethanol homogenate was filtered, the filtrate extracted with Skellyslov A, and the extract concentrated to a volume of 3 ml under reduced pressure. Concentrated alcoholic extracts were separated by

two dimensional paper chromatography, phenol (80) : water (20), (w/w) in the 1st direction and *n*-butanol (74) : acetic acid (19) : water (50), (v/v/v) in the 2nd direction. Compounds were located with the appropriate sprays: amino acids with ninhydrin in collidine spray of Levy and Chung (9), organic acids with a mixed indicator spray of 3 g brom-phenol blue and 1 g methyl red per liter of 95 % ethanol. Radioactive compounds were located by radioautograms made by exposing the chromatograms to "no-screen" x-ray film. Activity of each compound was measured directly on the paper using an end-window Geiger tube.

2,4-Dinitrophenylhydrazones were prepared according to a modified procedure described by Ranson<sup>4</sup>, in order to trap the  $\alpha$ -keto acids which would otherwise be lost during the preparation and the subsequent chromatographic analysis of the plant extract. Approximately 8 g of leaves, with the midribs removed, were exposed to C<sup>14</sup>O<sub>2</sub> in the absence of light for 10 minutes. The reaction was stopped by homogenization of the leaves with 20 ml of ice cold 5 N H<sub>2</sub>SO<sub>4</sub>. The homogenate was immediately filtered and the residue washed with 5 ml of 5 N H<sub>2</sub>SO<sub>4</sub>. Twenty-five ml of a saturated solution of 2,4-dinitrophenylhydrazine in 5 N H<sub>2</sub>SO<sub>4</sub> was then added to the filtrate and placed in the cold for 24 hours to permit the formation of the 2,4-dinitrophenylhydrazones. The hydrazones were extracted from the acid solution with three 30-ml portions of ethyl acetate. The ethyl acetate was then extracted with three 30-ml portions of ice cold 10 % Na<sub>2</sub>CO<sub>3</sub>. The combined 10 % Na<sub>2</sub>CO<sub>3</sub> extracts were adjusted to pH 1.0 with ice cold 5 N H<sub>2</sub>SO<sub>4</sub> and extracted with three 20-ml portions of ethyl acetate. The combined ethyl acetate extracts were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated to a volume of 2 ml under reduced pressure. The 2,4-dinitrophenylhydrazone derivatives were chromatographed on buffered paper by the method of Isherwood and Cruikshank (7) and identified by co-chromatography with authentic samples. Radioautograms were prepared as above. The activity of each compound was measured directly on the paper using an end-window Geiger tube.

The enzyme, phosphoenolpyruvate carboxylase, was prepared from acetone powder of tobacco leaves and assayed by the method described by Bandurski and Greiner (1). The acetone powder was prepared by homogenizing 50 g of tobacco leaves with 500 ml of acetone in a Waring blender at -20° C. The homogenate was immediately filtered through a Buchner

<sup>1</sup> Received August 7, 1958.

<sup>2</sup> This investigation was supported by a research grant from the Tobacco Industry Research Committee. The facilities of the Allan Hancock Foundation were generously provided.

<sup>3</sup> A preliminary report was presented at the meeting of the American Society of Plant Physiologists, August 26-30, 1956, University of Connecticut, Storrs, Connecticut.

<sup>4</sup> Personal communication.

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funnel and washed with 50 ml of  $-20^{\circ}\text{C}$  acetone. The powdered residue was dried over  $\text{PCl}_5$  under vacuum. The dried acetone powder was stored in a tightly sealed bottle at  $0^{\circ}\text{C}$ . The enzyme was prepared by extracting 0.2 g of the acetone powder with 5.0 ml of 0.005 M pH 7.5 TRIS buffer, in the cold. After centrifugation this extract was used directly.

### RESULTS AND DISCUSSION

Exposure of the tobacco leaves to  $\text{C}^{14}\text{O}_2$  in the dark for periods from 6 seconds to 5 hours, resulted in the sequential incorporation of the radioactive carbon into several compounds, principally organic and amino acids. Table I shows the total amount of radioactivity

TABLE I

RATE OF THE DARK FIXATION OF  $\text{C}^{14}\text{O}_2$  BY LEAVES OF NICOTIANA, TABACUM AND BRYOPHYLLUM CALYCINUM

TIME DARK EXPOSURE TO $\text{C}^{14}\text{O}_2$ (MIN)	CPM/MG OF LEAVES/MG $\text{BaCO}_3^*$	
	N. TABACUM	B. CALYCINUM
1	9	6
5	22	10
15	50	23
30	79	109
60	124	407
120	209	2,280

Leaves of tobacco and Bryophyllum were exposed independently to  $\text{C}^{14}\text{O}_2$  in the dark for the indicated time intervals. The reaction was terminated by homogenizing with boiling 80 % ethanol, and an aliquot of the homogenate assayed for radioactivity.

\* Samples were counted within a statistical error of 5 %.

incorporated by tobacco and Bryophyllum leaves exposed to  $\text{C}^{14}\text{O}_2$  in the dark for various intervals. It appears that tobacco can fix  $\text{CO}_2$  more rapidly in the initial stages of the dark carboxylation reaction. However, after the 1st 15 minutes the rate in the Bryophyllum seems to surpass that found in the tobacco leaves.

Malate and aspartate were the major detectable radioactive products on chromatograms of tobacco leaves exposed to  $\text{C}^{14}\text{O}_2$  for 6 seconds. However, traces of citrate were also observed. The amount of citrate accounted for less than 0.01 % of the total activity present. The percent of total activity found in malate and aspartate after successively longer periods of exposure to  $\text{C}^{14}\text{O}_2$  are plotted in figure 1. The extrapolation of these values to zero time strongly suggests the presence of a common precursor to malate and aspartate, probably oxaloacetate. To test this hypothesis, oxaloacetate was isolated and identified by co-chromatography as the 2,4-dinitrophenylhydrazone derivative from tobacco leaves exposed to  $\text{C}^{14}\text{O}_2$  in the dark for 10 minutes. It contained considerable radioactivity.

Since these results are similar to those found for succulent leaves (12, 13), the enzyme mediating the initial fixation of  $\text{CO}_2$ , phosphoenolpyruvate carboxylase, was extracted, and the data from a typical en-

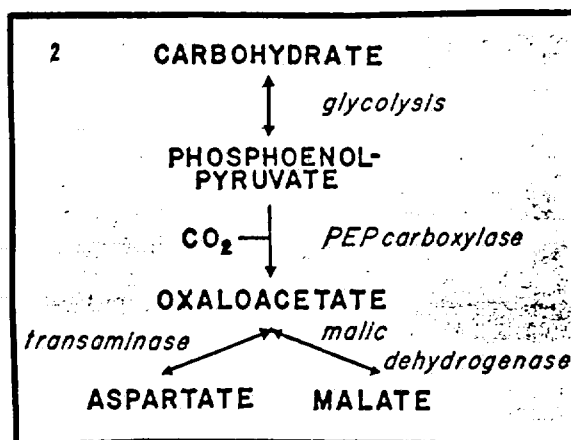
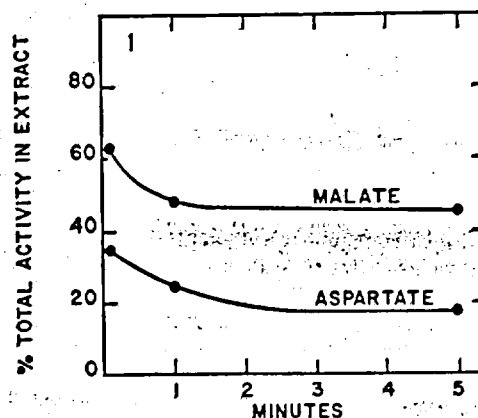


FIG. 1. Radioactivity of malate and aspartate expressed as percent total activity in extract as a function of time.

FIG. 2. Initial reactions involved in the dark fixation of  $\text{CO}_2$  by tobacco leaves.

zymatic assay are presented in table II. From these results it would appear that the initial carboxylation reaction is identical in both *Nicotiana tabacum* and *Bryophyllum calycinum*. It is suggested that figure 2 represents the initial reactions involved in the dark

TABLE II

PHOSPHOENOLPYRUVATE CARBOXYLASE ACTIVITY IN TRIS BUFFER EXTRACTS OF ACETONE POWDER PREPARED FROM TOBACCO LEAVES

CONDITION	CPM FIXED IN REACTION MIXTURE
Enzyme + phosphoenolpyruvate	10,600 $\pm$ 530
Enzyme — phosphoenolpyruvate	400 $\pm$ 20
Boiled + phosphoenolpyruvate	0
enzyme	

Each tube contained 60 micromoles TRIS hydrochloride pH 7.5, 20 micromoles  $\text{MgSO}_4$ , 100,000 cpm  $\text{NaH}^{14}\text{CO}_3$ , 0.2 ml enzyme, 6 micromoles phosphoenolpyruvate, total volume 1.5 ml. Incubated 60 minutes at  $37^{\circ}\text{C}$ . Reaction stopped with 0.1 ml 1 N HCl and the unreacted  $\text{C}^{14}\text{O}_2$  removed by bubbling  $\text{N}_2$  through the mixture. An 0.2-ml aliquot of the reaction mixture was counted with an end-window Geiger tube.

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fixation of CO<sub>2</sub> in tobacco leaves. The isolation of labeled malate from young tobacco leaves exposed to C<sup>14</sup>O<sub>2</sub> in the dark by Stutz and Burris (17) gives further evidence for the presence of a carboxylation reaction. Mazelis and Vennesland (10) have shown that the enzymes phosphoenolpyruvate carboxylase and phosphoenolpyruvate carboxykinase are widely distributed in plant tissues. These results suggest the general occurrence of a mechanism for the dark fixation of CO<sub>2</sub> in leaves of higher plants similar to those we have proposed for both *Bryophyllum calycinum* (12) and *Nicotiana tabacum*. Vickery and Puchler (21) have proposed the presence of a similar reaction to account for the accumulation of citrate in tobacco leaves cultured in bicarbonate solutions in the dark. Incorporation of bicarbonate into citrate could take place via an initial carboxylation reaction to form a 4-carbon dicarboxylic acid which subsequently condenses with acetate.

The suggestion has been made by Bradbeer et al (3) that the initial carboxylation in the dark CO<sub>2</sub> fixation is on ribulose diphosphate. This unstable  $\beta$ -keto intermediate is immediately cleaved to phosphoglycerate and is subsequently metabolized to the 3-carbon acceptor phosphoenolpyruvate. This compound is then carboxylated to form the 4-carbon dicarboxylic acid. We have not been able to identify labeled phosphoglycerate in our short term experiments in either tobacco or *Bryophyllum* leaves exposed to C<sup>14</sup>O<sub>2</sub> in the absence of light. However, after 5 minutes of dark fixation radioactivity can be detected in phosphoglycerate. It is possible that the age or the previous condition of the leaves could account for the differences in our observations.

Excised leaves of *Nicotiana tabacum* were exposed to C<sup>14</sup>O<sub>2</sub> in the dark for periods as long as 5 hours. A typical radioautograph is presented in figure 3. The rates at which the compounds incorporate the radio-carbon from C<sup>14</sup>O<sub>2</sub> are listed in table III. The radio-

TABLE III  
PRODUCTS FROM THE DARK FIXATION OF C<sup>14</sup>O<sub>2</sub>  
BY *N. TABACUM* LEAVES

COMPOUNDS	1 MINUTE	5 MINUTES	15 MINUTES	30 MINUTES
Malate	47.1	46.4	55.2	59.8
Citrate	12.1	11.6	3.6	5.3
Isocitrate	2.3	1.5	1.8	2.6
Succinate	1.9	4.7	5.8	4.5
Fumarate	0.3	1.0	1.1	1.1
Aspartate	23.9	17.4	13.2	8.0
Alanine	2.3	4.3	10.0	13.5
Glutamate	3.2	1.2	3.4	1.5
Serine	2.4	0.8	0.8	0.4
Glycine	0.6	0.2	0.4	0.2
Glutamine		Trace	1.1	1.3
Histidine			Trace	Trace
Proline			Trace	Trace
Threonine			Trace	Trace
Hydroxy-proline			Trace	Trace
Arginine			Trace	Trace

Concentrated alcoholic extracts of tobacco leaves exposed to C<sup>14</sup>O<sub>2</sub> for the indicated intervals were separated by 2-dimensional paper chromatography as described. Following location and identification of radioactive compounds by radioautography the activity in each compound was measured with an end-window Geiger tube.

Activities are expressed as percent of total activity counted on the chromatogram. Samples were counted within a statistical error of 5%. Those compounds indicated as "Trace" had less than 0.1% of the total activity.

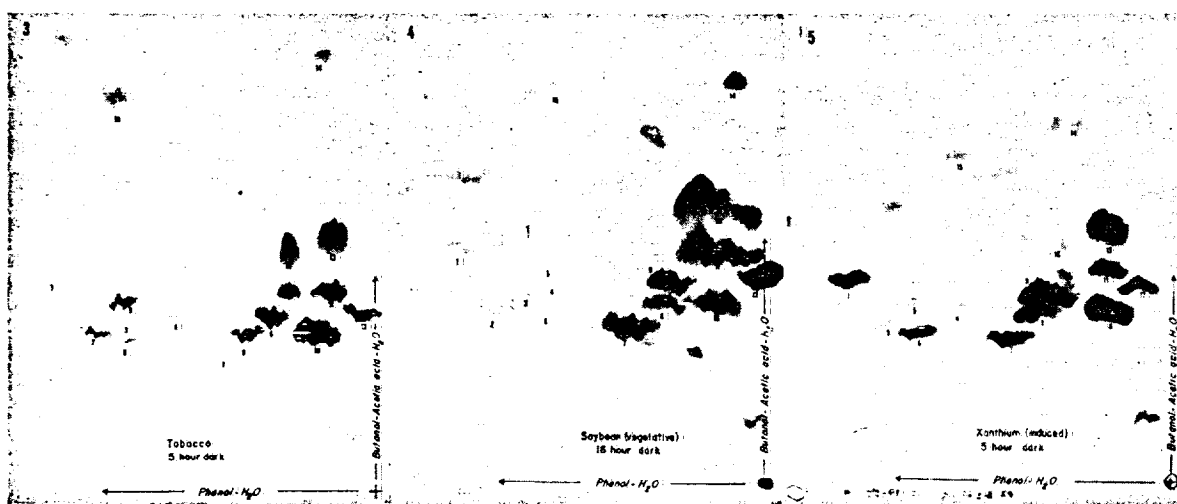


FIG. 3. Radioautogram of ethanol extract from tobacco leaf after 5 hours of dark C<sup>14</sup>O<sub>2</sub> fixation. Compounds which have been identified are: 1. proline, 2. arginine, 3. hydroxy-proline, 4. threonine, 6. glutamine, 7. asparagine, 8. serine-glycine, 9. glutamate, 10. aspartate, 11. unidentified ester of malic acid, 12. isocitrate-citrate, 13. malate, 14. fumarate, 15. succinate, 16. glycerate.

FIG. 4. Radioautogram of extracts from soybean showing the absence of carbohydrates and phosphorlated derivatives. Compounds identified are numbered as in figure 3.

FIG. 5. Radioautogram of extracts from xanthium showing the absence of carbohydrates and phosphorlated derivatives. Compounds identified are numbered as in figure 3.

activities are expressed as percent of total activity counted on the paper at given time intervals.

In addition to the listed compounds, the labile  $\alpha$ -keto acids, pyruvate, oxaloacetate and  $\alpha$ -ketoglutarate were also isolated and identified as their 2,4-dinitrophenylhydrazones. There was no detectable activity present in glyoxylate. We have been unable to detect any radioactivity associated with the carbohydrate or phosphorylated carbohydrate fractions in tobacco leaves exposed to  $C^{14}O_2$  in the dark for 5 hours. Sen and Leopold (14) report a rather significant carbohydrate fraction, 7 to 77%, of the  $C^{14}O_2$  fixed in the dark after 16 hours by Biloxi soybean, cockelbur, and Wintex barley. Our attempts to confirm the results of Sen and Leopold by exposing detached leaves of the above plants to  $C^{14}O_2$  for 16 hours have thus far been unsuccessful. Radioautographs of the extracts from soybean, cockelbur are shown in figure 4. It would appear in leaves and other organs of higher plants that light is necessary for the generation of the reducing power needed to reverse the glycolytic pathway. However, germinating castor beans (16) can incorporate  $C^{14}O_2$  into carbohydrates in the absence of light under conditions where fat is transformed into sugar.

The presence of an active Krebs cycle in photosynthesizing tissues has been an elusive problem for a long time. Recently, Smillie (15) has resolved this problem by preparing active cytoplasmic particles from green pea leaves, thus clearly establishing for the first time the operation of an active Krebs cycle in green leaves. We have been able to identify and isolate essentially all of the intermediates of the Krebs cycle with the exception of cis-aconitate. Although we have not demonstrated directly the enzymatic systems present in the cycle, the incorporation of  $C^{14}O_2$  into cycle acids and related amino acids suggests that the cycle is operative in tobacco leaves.

The results obtained by Vickery et al (20) in their studies on the metabolism of excised tobacco leaves in various culture media strongly support the operation of an active Krebs cycle. Malate, succinate, and fumarate were shown to be equally effective as precursors in the formation of citrate. With succinate as a substrate significant amounts of malate accumulated. A decrease in malate concentration was observed when fumarate was used. Vickery et al (20) feel that these observations are not compatible with the operation of the Krebs cycle since fumarate should be a mandatory intermediate in the conversion of succinate to malate. It is possible to formulate another hypothesis consistent with the operation of the Krebs cycle to explain the above results. If one considers that malate accumulation is controlled by the ability of the plant cell to transport this acid into a metabolically inactive area of the cell such as the vacuole, it is possible that the addition of fumarate in high concentrations could inhibit this transport mechanism resulting in a decreased concentration of malate. Preliminary experiments in our laboratory indicate that differential vacuolar transport systems are operative. This inhibition of malate accumulation by fumarate would not in any

way affect the metabolic pathway leading to the formation of citrate. Vickery et al (20) report that samples cultures in succinate did not accumulate any detectable fumarate. Therefore, succinate is converted to citrate and the accumulation of malate occurs in the absence of the inhibitory effects of abnormally high concentration of fumarate.

Glycolate did not incorporate radiocarbon in the dark when leaves were exposed to  $C^{14}O_2$  for as long as 5 hours in the absence of light. It was reported earlier by Benson and Calvin (2) that in *Chlorella*, *Scenedesmus* and barley leaves labeled glycolate was found only during photosynthesis. Claggett, Tolbert and Burris (4) demonstrated that glyoxylate is formed by the direct oxidation of glycolate in plants. Kenton and Mann (8) found that glyoxylate can be oxidized to oxalate in tobacco leaves. Our inability to find labeled glycolate, glyoxylate or oxalate in excised leaves of tobacco exposed to  $C^{14}O_2$  in the dark would appear to be in complete agreement with the known biosynthetic pathways of these compounds in leaves of higher plants. Although oxalate is one of the major acid components of tobacco leaves (19), we have been unable to demonstrate the incorporation of radiocarbon from  $C^{14}O_2$  into this acid after 5 hours in the absence of light.

Serine incorporated  $C^{14}$  prior to glycine in tobacco leaves, as has been observed with *Bryophyllum* (13). Serine has been demonstrated to be precursor to glycine in animal tissues (6). Whether serine is synthesized in higher plants by a phosphorylated or non-phosphorylated pathway cannot be determined at the present time, since we have found both glycinate and phosphoglycerate labeled in the plant extracts.

Tolbert and Cohan (18) have shown that the major products formed from glycolate in barley and wheat leaves are glycine, serine and an unknown compound. There is a direct conversion of the glycolate to glycine and to the carboxyl and  $\alpha$ -carbon of serine. The  $\beta$ -carbon of serine is formed from the  $\alpha$ -carbon of glycolate. This evidence clearly establishes a direct biosynthetic pathway for the synthesis of glycine from glycolate. Therefore, it appears that there are two separate pathways present in leaves of higher plants for the biosynthesis of serine and glycine. It is difficult to assess the relative importance of the two pathways. Certainly in the dark, where  $CO_2$  fixation in glycolate is not observed, radioactive serine arises from a 3-carbon precursor.

The striking qualitative similarity of the metabolic products involved with the dark fixation of  $CO_2$  in excised leaves of *Nicotiana tabacum* and *Bryophyllum calycinum* are apparent from these studies. Although the rate of the initial  $CO_2$  fixation in the absence of light is comparable and the mechanism of the initial carboxylation reaction appears to be identical, the succulents appear to have a unique mechanism for the storage of the synthesized organic acids which might account for the accumulation of a large quantity of the fixed carbon dioxide. We are not able to account for the difference at this time.

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## SUMMARY

1. Excised leaves of *N. tabacum* have the ability to incorporate C<sup>14</sup>O<sub>2</sub> in the dark into several organic and amino acids. These compounds have been identified by paper chromatography and radioautography.

2. The initial rate of dark CO<sub>2</sub> fixation in tobacco is more rapid than that of the succulent *B. calycinum*. However, the total amount of CO<sub>2</sub> fixed by tobacco after extended periods is much less.

3. The initial fixation of C<sup>14</sup>O<sub>2</sub> appears to be mediated by the enzyme phosphoenolpyruvate carboxylase. The presence of this enzyme in tobacco leaves has been established.

4. The pattern of organic and amino acids which incorporate C<sup>14</sup>O<sub>2</sub> suggest the operation of the Krebs cycle and concomitant transaminations. Serine seems to be the precursor to glycine in the dark.

5. No carbohydrates or phosphorylated sugars are labeled after extended periods of dark incorporation of C<sup>14</sup>O<sub>2</sub>. These findings suggest that light is needed to furnish the reducing power to reverse glycolytic processes.

We wish to acknowledge the valuable suggestions and assistance of Dr. John L. Webb in the preparation of this manuscript.

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## DARK FIXATION OF CO<sub>2</sub> BY SUCCULENT LEAVES: CONSERVATION OF THE DARK FIXED CO<sub>2</sub> UNDER DIURNAL CONDITIONS<sup>1,2</sup>

GEORGE KUNITAKE AND PAUL SALTMAN

DEPARTMENT OF BIOCHEMISTRY AND NUTRITION, SCHOOL OF MEDICINE,  
UNIVERSITY OF SOUTHERN CALIFORNIA, LOS ANGELES 7, CALIFORNIA

The characteristic ability of the succulents to incorporate a large net amount of CO<sub>2</sub> in the form of organic acids has been recognized for a long time (9). Although the biochemical mechanisms of this phenomenon have received a great deal of attention, the physiological role has not been fully understood. In the course of our studies on the pathways of the dark fixation of CO<sub>2</sub> (6, 7), it was observed that the C<sup>14</sup>O<sub>2</sub> incorporated in the dark was not lost in the subsequent light period but appeared to be utilized directly in the photosynthesis. From these and other considerations we are impressed with a possibility that the special features of Crassulacean acid metabolism have an adaptive advantage.

### MATERIALS AND METHODS

A random sample of young *Bryophyllum calycinum* Salisb. leaves (approximately 2 to 3 cm long, wet weight 0.5 to 0.6 g), picked from the apex of the plant was placed in the apparatus described in a previous communication (6) and permitted to fix C<sup>14</sup>O<sub>2</sub> (generated from 4.6 mg of BaC<sup>14</sup>O<sub>3</sub>, specific activity 120 c/mg) for 30 minutes in the dark. Two of the leaves were homogenized immediately with boiling 80% ethanol and an aliquot counted for determination of total activity. All samples were counted at infinite thinness using a Micromil gas flow counter. The total homogenate was then filtered

through Whatman no. 1 paper, washed three times with small portions of boiling 80% ethanol, and an aliquot of the total filtrate analyzed by paper chromatography as indicated below. The remaining leaves were removed, placed on moist filter paper, and illuminated with a photoflood lamp (Photospot R.S.P. 2, G.E. 115 to 120 v) placed 31 to 32 inches away. Cool air circulated over the surface of the leaves with a fan to prevent over-heating. At no time did the temperature at the surface of the leaves exceed 28° C. At various intervals pairs of leaves were removed and homogenized in boiling 80% ethanol. The determination of the total activity in the homogenate and the chromatographic analysis of the filtrate was carried out as described below. As a control, one pair of leaves was exposed 30 minutes to C<sup>14</sup>O<sub>2</sub> in the dark, flushed free of C<sup>14</sup>O<sub>2</sub>, kept in the dark for 120 minutes, and homogenized in boiling 80% ethanol.

Identification of the labeled photosynthetic and dark products was made on aliquots of the 80% ethanol soluble fractions. This was accomplished by two dimensional chromatography on Whatman no. 1 filter paper using phenol (80): water (20) (w/w) in the 1st direction and *n*-butanol (74): acetic acid (19): water (50) (v/v/v) in the 2nd. Radioautographs were made with "no-screen" x-ray film. In all experiments, non-labeled glucose and sucrose were co-chromatographed with the extract. Identification of the radioactive compounds was made by superposition. Activity was determined directly on the paper with an end-window Geiger tube.

An alternate technique was employed to ascertain whether the C<sup>14</sup>O<sub>2</sub> fixed in the dark was released to

<sup>1</sup> Received April 16, 1958.

<sup>2</sup> This investigation was supported by a research grant from the Tobacco Industry Research Committee. The facilities of the Allan Hancock Foundation were generously provided.

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the atmosphere during the subsequent light period. Matched pairs of leaves were exposed to  $C^{14}O_2$  in the dark for 60 minutes. As a control, one pair was immediately homogenized in boiling 80 % ethanol, and the radioactivity determined on an aliquot. The other pair of leaves was placed in a glass chamber and illuminated with three 120-watt incandescent lamps placed 24 inches from the surface of the leaves. During the illumination, moist air was flushed through the chamber and the  $CO_2$  was trapped in 1 N NaOH, precipitated as  $BaCO_3$ , and counted with a gas flow Geiger tube.

Experiments were designed to test the effect of previous conditions of light or dark on the subsequent photosynthetic  $CO_2$  incorporation. Intact plants were placed either in a completely dark room for 17 to 24 hours or in direct sunlight. At the beginning of the experiment matched pairs of leaves were removed, placed in the glass illumination chamber, exposed to  $C^{14}O_2$ , and allowed to photosynthesize for 30 minutes. The leaves were then homogenized in boiling 80 % ethanol and the total activity measured on an aliquot as described above.

### RESULTS AND DISCUSSION

The total amount of radioactivity retained by the leaves at each time interval of illumination after the dark incorporation of  $C^{14}O_2$  is presented in table I. These data represent the averages of three experiments. A most interesting finding is that the total amount of radiocarbon incorporated in the dark period remained constant during the subsequent light period as well as during the dark control.

In our previous research concerning the dark metabolism of  $C^{14}O_2$  in *Bryophyllum* (7) we demonstrated that no detectable activity was incorporated into either the carbohydrates or their phosphorylated derivatives, all activity being associated with the organic and amino acids. Chromatographic analysis of the extract from the dark control confirmed this. However, when the leaves were transferred to light in the absence of exogenous  $C^{14}O_2$  significant activity was associated with the sucrose from the 80 % ethanol soluble fraction. Figure 1 presents the radioautographs from the dark and light extracts. It is clear that upon exposure of the leaf to light, not only was there a conservation of the  $C^{14}O_2$  fixed in the dark, but that  $CO_2$  was utilized in the formation of photosynthetic products.

TABLE I  
RADIOACTIVITY RETAINED BY *BRYOPHYLLUM* LEAVES  
AFTER ILLUMINATION

Duration of light	0	10	30	60	120	(120) Dark control
Total activity in homogenate* (cpm/mg leaf)	737	781	706	750	813	745

\* Samples were counted within a statistical error of 5%.

TABLE II  
LOSS OF DARK FIXED  $C^{14}O_2$  DURING THE SUBSEQUENT  
LIGHT PERIOD

EXPERIMENT	TOTAL ACTIVITY FIXED IN DARK (CPM)	TOTAL FREE $C^{14}O_2$ TRAPPED AS $BaCO_3$ (CPM)	PERCENT TOTAL ACTIVITY LOST
I	$2.4 \times 10^5$	$5.4 \times 10^5$	2.2
II	$5.0 \times 10^5$	$9.1 \times 10^5$	1.8

Evidence for this conservation of dark-fixed  $CO_2$  during photosynthesis was obtained by the 2nd technique in which the  $CO_2$  was trapped during the process of photosynthesis. The data from typical experiments are shown in table II. It is clear that less than 3% of the total activity accumulated by the leaves was in equilibrium with the external atmosphere. This experiment indicates that the  $CO_2$  incorporated during the dark was efficiently retained, and utilized during subsequent photosynthesis in the leaf. Similar results have been observed with a marine flagellate, *Dunaliella euehlora* by Ryther (5). Ryther interprets his data as evidence for a preferential utilization of endogenous  $CO_2$ .

The ability of succulents to incorporate large amounts of  $CO_2$  in the dark, and subsequently utilize this  $CO_2$  for photosynthesis is a possible biochemical adaptation to arid conditions. The thick spongy leaves and stems with rather impermeable cuticles are useful anatomical structures for water conservation. Since photosynthetic activity takes place at those times when water loss is maximal, re-utilization by the leaf of the dark incorporated  $CO_2$  would tend to minimize the need for opening the stomata to permit gas exchange, and might aid in the conservation of water. Physiological observations of Loftfield (2) support this hypothesis. He demonstrated that, unlike non-succulents, the stomata of the succulents remained closed during the day, but opened at night to permit gas exchange.

Thomas et al (10) have shown that under normal atmospheric  $CO_2$  concentration (0.03 %), light deacidification takes place in succulent leaves. However, when the leaves are exposed to high concentrations of  $CO_2$  (5 to 10 %), this light deacidification can be retarded, and under certain conditions light acidification can be induced. The only known mechanism for the synthesis of carbohydrates from dicarboxylic acids involves decarboxylation and reversal of glycolysis. The observations of Thomas et al (10) could be interpreted as evidence for the preferential carboxylation of ribulose diphosphate as compared to phosphoenol-pyruvate. During the light period following dark acidification  $CO_2$  liberated via decarboxylation immediately enters into the light  $CO_2$  fixation mechanism. However, when the ribulose diphosphate pathway for  $CO_2$  fixation is saturated at high  $CO_2$  concentrations in the light, the acidification system involving phosphoenol-pyruvate carboxylase can be utilized.

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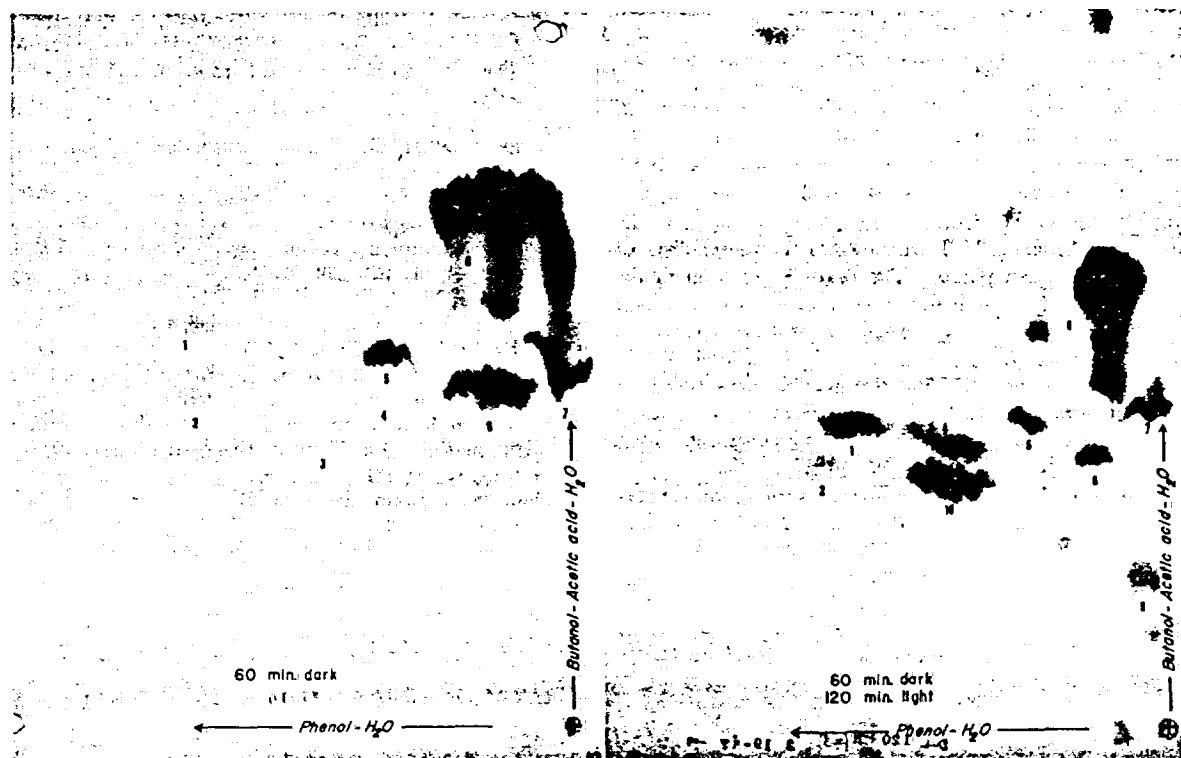


FIG. 1. Radioautograms of two dimensional paper chromatograms of extracts from 60-minute dark fixation of  $C^{14}O_2$ , and 60-minute dark fixation of  $C^{14}O_2$  followed by 120-minute photosynthesis in absence of  $C^{14}O_2$ . Compounds indicated are: 1. alanine, 2. glutamine, 3. asparagine, 4. glycine-serine, 5. glutamate, 6. aspartate, 7. citrate-isocitrate, 8. malate, 9. phosphoglycerate, and 10. sucrose.

The effects of previous exposure of the leaves to light or dark on the total photosynthetic  $CO_2$  fixation are presented in table III. The experiments were designed so that the leaves of the plants pretreated in the light would be depleted of most of their organic acids. These data are very similar to those presented by Sen and Leopold (8). There are several interpretations that can be made from these results. Sen and Leopold favor the view that there is some temporary impairment of the mechanisms of photosynthesis during the previous exposure to dark. This impairment was manifested in the diminution of the  $C^{14}O_2$  incorporated. Another interpretation is possible if one postulates that the rates of photosynthesis subsequent to either pretreatment are approxi-

mately the same. The  $CO_2$  fixed during the dark periods would provide a considerable store of endogenous  $CO_2$  which was preferentially utilized. This would result in the decreased utilization of the exogenous  $CO_2$ . It is well known from the work of Pucher et al (3, 4) that there is an inverse quantitative relationship between total titratable acids and net carbohydrate stores in succulent leaves subjected to diurnal conditions.

The known pathways for the interconversion of Krebs cycle organic acids and carbohydrates depend upon decarboxylation and the reversal of glycolysis. The labeling experiments of Varner and Burrell (11) and Gibbs (1) completely support this hypothesis. This evidence in conjunction with the experiments presented in this paper indicates that the  $CO_2$  derived from decarboxylation of the organic acids is retained by the cell and metabolized via the normal photosynthetic mechanisms.

#### SUMMARY

Detached leaves of *Bryophyllum calycinum* are able to fix, in the dark, large amounts of  $C^{14}O_2$  in the form of organic and amino acids. When these leaves are exposed to light in the absence of  $C^{14}O_2$ , radioactivity is found in the carbohydrate fraction. The dark fixed  $C^{14}O_2$  is efficiently conserved and metabolized during subsequent photosynthetic reactions.

TABLE III  
PHOTOSYNTHETIC  $C^{14}O_2$  FIXATION LEAVES PRETREATED  
IN DARK OR IN LIGHT

PRETREATED IN LIGHT		PRETREATED IN DARK		RATIO LIGHT/DARK
HOURS OF PRE- TREATMENT	TOTAL CPM	HOURS OF PRE- TREATMENT	TOTAL CPM	
8	$38.2 \times 10^5$	17	$7.7 \times 10^5$	5
8	$23.9 \times 10^5$	24	$1.9 \times 10^5$	12

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Committee:

Dr. Kotin, Chairman  
Dr. Jacobson  
Dr. Wilson

Cf. #6  
Activated 10/1/54  
Renewed 10/1/55  
Cf. #142  
Activated 1/1/57  
Renewed 1/1/58

TOBACCO INDUSTRY RESEARCH COMMITTEE

150 East Forty Second Street New York 17, N.Y.

Application For Research Grant

Date: August 1, 1960

1. Name of Investigator: Paul D. Saltman, Ph.D.
2. Title: Associate Professor of Biochemistry and Nutrition
3. Institution & Address: School of Medicine, University of Southern California  
Los Angeles 7, California
4. Project or Subject: The Mechanisms of Photosynthetic Reduction.
5. Detailed Plan of Procedure:

Our primary goal in this research is to elucidate the specific requirements for chemical energy and reducing power necessary to effect the reductive fixation of  $C^{14}O_2$  by cell-free tobacco leaf systems in the dark. There is little doubt that ATP and TPNH can be generated by illuminated chloroplasts. However, preliminary experiments in our laboratory have demonstrated that these compounds cannot replace light in a cell-free system capable of carrying out photosynthesis. If we are able to mimic the patterns of the photosynthetic reduction of  $C^{14}O_2$  in the dark, we will have a more intimate understanding of the mechanisms for the conversion of light energy to reducing power and chemical energy by higher plants.

During the past year, Dr. Joshi and I have developed a cell-free preparation of tobacco leaves containing broken chloroplasts, mitochondria, microsomes, and soluble cytoplasm which fixes  $C^{14}O_2$  in the dark by the identical metabolic pathways as the intact leaf. This preparation when illuminated carries out photosynthetic  $C^{14}O_2$  fixation. Chromatographic analysis of the dark system, with the addition of ATP, TPNH, and many other co-factors, reveals the absence of any radioactivity in the carbohydrates, despite several thousand counts per minute in the organic and amino acids. It is our plan to attempt to trap labile or transient intermediates generated in the photo-reduction steps by illuminating the system at low temperatures, turning off the light, adding  $C^{14}O_2$ , and measuring the radioactivity incorporated into the sugar fraction in the dark. Only in the presence of light-generated reducing power will such events take place. If this procedure is successful, we will attempt the direct isolation of the reducing compounds by various chemical and physical procedures. If this approach meets with negative results, we will concentrate on a program utilizing specific inhibitors and antimetabolites to block the light reactions without affecting the dark pathways and thus learn more of the nature of the light requiring steps.

1003541074

6. Budget Plan:

Salaries	4,200
Expendable Supplies	1,200
Permanent Equipment	--
Overhead (15%)	870
Other (Travel)	400
Total	\$6,670

7. Anticipated Duration of Work: The budget presented above will suffice for our first year's program. If the results warrant extension of our research, we will seek continued support for a longer period.

8. Facilities and Staff Available: We have a well-equipped biochemical laboratory with facilities for the preparation of homogenates, enzymatic isolations, etc. We also have an adequate isotope laboratory and have had extensive experience with chromatography and radioautography.

We have excellent liaison with the plant physiologists at California Institute of Technology, and have their full cooperation in the growing of plants in their greenhouses. Dr. G. V. Joshi is a Visiting Professor from Wilson College, Bombay, India. He has broad experience and several publications in the field of plant metabolism and CO<sub>2</sub> fixation. Dr. P. Saltman has engaged in an active program of research on the pathways of the dark and light fixation of CO<sub>2</sub> by plants for the past seven years. An excellent graduate student, Mr. Robert Gee, has entered the department this summer and is interested in working on this problem. We are a small group, but highly activated. Most of the preliminary methodology is in hand and we are ready to proceed with the major problem.

9. Additional Requirements:

10. Additional Information: As indicated above, the amount requested of the TIRC should be adequate for our first year. We hope our success is such that we can seek renewal for a longer period.

The salary of Dr. Joshi is being paid by the National Research Council. He is spending two years in our laboratory as a Post-doctoral Fellow of that organization.

No other support is available for this project.

Signature \_\_\_\_\_

Director of Project

\_\_\_\_\_  
Business Officer of Institution

RCH Note: This is an advance working copy.  
The official copy bearing signatures  
of applicant and university officer  
is enroute.

1003541075

TOBACCO INDUSTRY RESEARCH COMMITTEE  
350 FIFTH AVENUE NEW YORK 1, N. Y.

Re - Application For Research Grant

# 82 A

Date: April 28, 1955

1. Name of Investigator: (a) Milton S. Saslaw, M.D.  
2. Anticipated Director of: (b) Murray M. Streitfeld, Ph.D.

3. Title: (a) Director of Medical Research Laboratory  
(b) Bacteriologist, Autoclave  
(c) Dry Heat Oven

3. Institution & Address: National Children's Cardiac Hospital  
4250 West Flagler Street  
Miami 34, Florida

4. Project or Subject: "Effects of Smoking on Beta Hemolytic Streptococci"

Studies will be made to determine: (a) (b) (c) (d) (e) (f) (g) (h) (i) (j) (k) (l) (m) (n) (o) (p) (q) (r) (s) (t) (u) (v) (w) (x) (y) (z) (aa) (ab) (ac) (ad) (ae) (af) (ag) (ah) (ai) (aj) (ak) (al) (am) (an) (ao) (ap) (aq) (ar) (as) (at) (au) (av) (aw) (ax) (ay) (az) (ba) (bb) (bc) (bd) (be) (bf) (bg) (bh) (bi) (bj) (bk) (bl) (bm) (bn) (bo) (bp) (bq) (br) (bs) (bt) (bu) (bv) (bw) (bx) (by) (bz) (ca) (cb) (cc) (cd) (ce) (cf) (cg) (ch) (ci) (cj) (ck) (cl) (cm) (cn) (co) (cp) (cq) (cr) (cs) (ct) (cu) (cv) (cw) (cx) (cy) (cz) (da) (db) (dc) (dd) (de) (df) (dg) (dh) (di) (dj) (dk) (dl) (dm) (dn) (do) (dp) (dq) (dr) (ds) (dt) (du) (dv) (dw) (dx) (dy) (dz) (ea) (eb) (ec) (ed) (ee) (ef) (eg) (eh) (ei) (ej) (ek) (el) (em) (en) (eo) (ep) (eq) (er) (es) (et) (eu) (ev) (ew) (ex) (ey) (ez) (fa) (fb) (fc) (fd) (fe) (ff) (fg) (fh) (fi) (fj) (fk) (fl) (fm) (fn) (fo) (fp) (fq) (fr) (fs) (ft) (fu) (fv) (fw) (fx) (fy) (fz) (ga) (gb) (gc) (gd) (ge) (gf) (gg) (gh) (gi) (gj) (gk) (gl) (gm) (gn) (go) (gp) (gq) (gr) (gs) (gt) (gu) (gv) (gw) (gx) (gy) (gz) (ha) (hb) (hc) (hd) (he) (hf) (hg) (hh) (hi) (hj) (hk) (hl) (hm) (hn) (ho) (hp) (hq) (hr) (hs) (ht) (hu) (hv) (hw) (hx) (hy) (hz) (ia) (ib) (ic) (id) (ie) (if) (ig) (ih) (ii) (ij) (ik) (il) (im) (in) (io) (ip) (iq) (ir) (is) (it) (iu) (iv) (iw) (ix) (iy) (iz) (ja) (jb) (jc) (jd) (je) (jf) (jg) (jh) (ji) (jj) (jk) (jl) (jm) (jn) (jo) (jp) (jq) (jr) (js) (jt) (ju) (jv) (jw) (jx) (jy) (jz) (ka) (kb) (kc) (kd) (ke) (kf) (kg) (kh) (ki) (kj) (kk) (kl) (km) (kn) (ko) (kp) (kq) (kr) (ks) (kt) (ku) (kv) (kw) (kx) (ky) (kz) (la) (lb) (lc) (ld) (le) (lf) (lg) (lh) (li) (lj) (lk) (ll) (lm) (ln) (lo) (lp) (lq) (lr) (ls) (lt) (lu) (lv) (lw) (lx) (ly) (lz) (ma) (mb) (mc) (md) (me) (mf) (mg) (mh) (mi) (mj) (mk) (ml) (mm) (mn) (mo) (mp) (mq) (mr) (ms) (mt) (mu) (mv) (mw) (mx) (my) (mz) (na) (nb) (nc) (nd) (ne) (nf) (ng) (nh) (ni) (nj) (nk) (nl) (nm) (nn) (no) (np) (nq) (nr) (ns) (nt) (nu) (nv) (nw) (nx) (ny) (nz) (oa) (ob) (oc) (od) (oe) (of) (og) (oh) (oi) (oj) (ok) (ol) (om) (on) (oo) (op) (oq) (or) (os) (ot) (ou) (ov) (ow) (ox) (oy) (oz) (pa) (pb) (pc) (pd) (pe) (pf) (pg) (ph) (pi) (pj) (pk) (pl) (pm) (pn) (po) (pp) (pq) (pr) (ps) (pt) (pu) (pv) (pw) (px) (py) (pz) (qa) (qb) (qc) (qd) (qe) (qf) (qg) (qh) (qi) (qj) (qk) (ql) (qm) (qn) (qo) (qp) (qq) (qr) (qs) (qt) (qu) (qv) (qw) (qx) (qy) (qz) (ra) (rb) (rc) (rd) (re) (rf) (rg) (rh) (ri) (rj) (rk) (rl) (rm) (rn) (ro) (rp) (rq) (rr) (rs) (rt) (ru) (rv) (rw) (rx) (ry) (rz) (sa) (sb) (sc) (sd) (se) (sf) (sg) (sh) (si) (sj) (sk) (sl) (sm) (sn) (so) (sp) (sq) (sr) (ss) (st) (su) (sv) (sw) (sx) (sy) (sz) (ta) (tb) (tc) (td) (te) (tf) (tg) (th) (ti) (tj) (tk) (tl) (tm) (tn) (to) (tp) (tq) (tr) (ts) (tu) (tv) (tw) (tx) (ty) (tz) (ua) (ub) (uc) (ud) (ue) (uf) (ug) (uh) (ui) (uj) (uk) (ul) (um) (un) (uo) (up) (uq) (ur) (us) (ut) (uu) (uv) (uw) (ux) (uy) (uz) (va) (vb) (vc) (vd) (ve) (vf) (vg) (vh) (vi) (vj) (vk) (vl) (vm) (vn) (vo) (vp) (vq) (vr) (vs) (vt) (vu) (vv) (vw) (vx) (vy) (vz) (wa) (wb) (wc) (wd) (we) (wf) (wg) (wh) (wi) (wj) (wk) (wl) (wm) (wn) (wo) (wp) (wq) (wr) (ws) (wt) (wu) (wv) (ww) (wx) (wy) (wz) (xa) (xb) (xc) (xd) (xe) (xf) (xg) (xh) (xi) (xj) (xk) (xl) (xm) (xn) (xo) (xp) (xq) (xr) (xs) (xt) (xu) (xv) (xw) (xx) (xy) (xz) (ya) (yb) (yc) (yd) (ye) (yf) (yg) (yh) (yi) (yj) (yk) (yl) (ym) (yn) (yo) (yp) (yq) (yr) (ys) (yt) (yu) (yv) (yw) (yx) (yy) (yz) (za) (zb) (zc) (zd) (ze) (zf) (zg) (zh) (zi) (zj) (zk) (zl) (zm) (zn) (zo) (zp) (zq) (zr) (zs) (zt) (zu) (zv) (zw) (zx) (zy) (zz)

5. Detailed Plan of Procedure (Use reverse side if additional space is needed):  
Studies (1,2,3) during the past three years have established the presence of Group A beta hemolytic streptococci in the throats of 37.9 percent of school children in Dade County, Florida. The proposed study will determine the extent to which this rate applies to adults, and how it is influenced by smoking.

The plan of procedure is as follows:  
A. Throat cultures will be taken from smokers and non-smokers, in sufficient numbers to provide adequate statistical data. All beta hemolytic streptococcal isolates will be grouped and typed.

B. Blood samples will be drawn simultaneously from each subject for antistreptolysin O determinations.

C. Smoking habits will be determined in each case, considering the following factors:

- (a) Extent of Smoking - light or heavy smoker or non-smoker
- (b) Type of Smoking - cigarette, cigar or pipe
- (c) Filtered vs. non-filtered cigarettes
- (d) Duration of smoking habit
- (e) Inhalation vs. non-inhalation
- (f) Interval between taking of last smoke and throat culture

Signature: /s/ Milton S. Saslaw, M.D.  
Director of Project

/s/ J. J. Smith, M.D., Secy.  
Business Officer of the Institution

1003541076

5. Detailed Plan of Procedure (continued)

D. Various tobacco and smoke products will be investigated for their effects on the growth of beta hemolytic streptococci in vitro.

E. Findings will be evaluated and their significance indicated.

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(1) Saslaw, M.S. and Steitfeld, M.M.: Group A Beta Hemolytic Streptococci and Rheumatic Fever in Miami, Fla., Pub. Health Repts. 69: 877-822 (Sept.) 1954.

(2) Saslaw, M.S., Streitfeld, M.M. and Doff, S.D.: Group A Beta Hemolytic Streptococci and Rheumatic Fever in Miami, Fla.: Bacteriologic and Serologic Studies from October, 1953 through May, 1954. (in manuscript)

(3) Saslaw, M.S., Streitfeld, M.M., and Williams, E., Jr.: Use of An Antistreptolysin O Index for Comparison of Large Samples: Experience in Miami, Florida, Clin. Res. Proc. 3: 77 (Feb.) 1955

1003541077

# 6. Budget Plan: (1st year)

Salaries	\$5,860.00
Expendable Supplies	1,400.00
Permanent Equipment	640.00
Overhead	750.00
Other	
<b>Total</b>	<b>\$8,650.00</b>

7. Anticipated Duration of Work: **Wilton S. Saslaw, M.D.**  
(b) **3.2 years** Streittfeld, Ph.D.

8. Facilities and Staff Available: **Facilities:** (a) Bacteriology Laboratory (e) pH meter  
(b) Autoclave (f) Incubators  
(c) Dry Heat Oven (g) Arnold Sterilizer  
(d) Water-baths (h) Deep Freeze and Refrigerators  
**Staff:** (a) Milton S. Saslaw, M.D.  
(b) Murray M. Streittfeld, Ph.D.  
(c) Ruth Rosen (Technician)

9. Additional Requirements: **1. Technician (additional).....\$3,000.00**  
**2. Clerical Help ..... 2,860.00**

**3. Expendable Supplies:**  
(a) Culture Media .....300.00  
(b) Additional Glassware.....200.00  
(c) Sheep's Blood.....200.00  
(d) Streptolysin O .....300.00  
(e) Postage, stationery, forms, etc..... 400.00  
**4. Other Publications, travel to source material**

10. Additional Information (Including relation of work to other projects and other sources of supply):

Studies (1,2,3) and conventions ..... 750.00  
A beta hemolytic streptococcus in the throats of 1.9 percent of school children in Lake County, Florida. The present study will determine the extent to which this rate applies to adults, and how it is influenced by smoking.  
A general study of streptococcal patterns is currently under way. This is supported by the National Children's Cardiac Hospital, Florida State Board of Health, and a research grant from the United State Public Health Service. The request submitted herewith will permit expansion of the program to include the effects of smoking and tobacco products on the prevalence and growth of beta hemolytic streptococci.

2. Blood smears will be drawn simultaneously from each subject for anti-streptolysin O titrations.

3. Smears smears will be obtained in each case, considering the following factors:

- Effect of smoking - light or heavy smoker or non-smoker
- Type of smoking - cigarette, pipe or pipe
- Filtered and unfiltered cigarettes
- Amount of smoking, daily
- Interval between taking of last smoke and throat culture

Signature /s/ Milton S. Saslaw, M.D.  
Director of Project

/s/ J. Jacobs, Exec. Secy.  
Business Officer of the Institution

TOBACCO INDUSTRY RESEARCH COMMITTEE  
350 FIFTH AVENUE NEW YORK 1, N. Y.

Application For Research Grant

Date: **October 1, 1954**

1. Name of Investigator: **G. W. H. Schepers, M.D., D.Sc.**

2. Title: **Director**

3. Institution  
& Address: **The Saranac Laboratory  
7 Church Street  
Saranac Lake, New York**

4. Project or Subject:

**ENVIRONMENTAL PULMONARY CARCINOGENESIS. The co-carcinogenic potentialities of inhaled tobacco smoke in relation to beryllium-provoked lung cancer of the rat.**

5. Detailed Plan of Procedure (Use reverse side if additional space is needed):

- (a) See attached explanatory memorandum.
- (b) The Saranac Laboratory has perfected a technique of producing lung cancer in rats by means of beryllium aerosol inhalation.
- (c) It is proposed to examine the co-carcinogenicity of tobacco smoke with beryllium sulphate in rats.
- (d) The incidence and characteristics of pulmonary carcinoma will be studied in 500 rats which will be subjected to detailed histological and biochemical analysis.
- (e) Special precautions need to be taken to safeguard personnel against beryllium toxicity.

Signature

Director of Project

**G. W. H. Schepers, M.D., D.Sc.**  
Director, The Saranac Laboratory  
and as

President, The Tobacco Industry Research Committee  
Laboratory Board of

1003541079

TOBACCO INDUSTRY RESEARCH COMMITTEE  
350 FIFTH AVENUE NEW YORK 1, N. Y.

6. Budget Plan:

2 years

Salaries	\$21,120.
Expendable Supplies	14,465
Permanent Equipment	3,500.
Overhead	9,771.
Other	500.

Date: October 1, 1952 Total \$49,356

7. Anticipated Duration of Work: G. W. Schepers, M.D., D.Sc.  
24 months

8. Facilities and Staff Available:

See attached memorandum

The Saranac Laboratory  
7 Grand Street  
Saranac Lake, New York

9. Additional Requirements:

EXPERIMENTAL VOLUNTARY SARCINOMATOSIS. The dissemination of experimental tobacco smoke in relation to lung cancer. A liberal gratis source of supply of tobacco in the form in which it is currently being consumed by the public.

10. Additional Information (Including relation of work to other projects and other sources of supply):

- (a) See attached memorandum.
- (b) The work will be based on and amplify studies which have been conducted by The Saranac Laboratory during the past 4 years by means of grants-in-aid from, and to extend the co-susceptibility of tobacco smoke with lung cancer in rats.
- (c) The work is funded by The Damon Runyon Memorial Fund for Cancer Research, by studies by the American Cancer Society, detailed histological and molecular studies by The U. S. Atomic Energy Commission.
- (d) Special precautions need to be taken to safeguard personnel against lung cancer.

Signature \_\_\_\_\_

Director of Project

G. W. H. Schepers, M.D., D.Sc.  
Director, The Saranac Laboratory  
and as

Executive Vice-President of The Saranac  
Laboratory Board of  
Trustees

1003541080

## I. RATIONALE

The Saranac Laboratory has pioneered research into the problems of chest diseases during the past seventy years. During the past thirty years it has concentrated more particularly on the industrial diseases. The long range perspective gained by the eleven hundred experiments already conducted by the Laboratory and the innumerable clinical diagnostic problems it has encountered have brought into sharp focus the growing problem of lung cancer. The Laboratory has, moreover, through its extensive surveys of industrial environmental hazards, become acutely conscious of the question whether the growing incidence of lung cancer does indeed bear some specific causal relationship to the multiplicity of noxious substances which form part of the respiratory milieu of the civilized human being.

In an attempt to provide a positive answer for some of the questions relating to environmental pulmonary carcinogenesis the Laboratory has conducted statistical surveys to probe the relationship between specific industrial exposures and lung cancer and has conducted a series of experiments in an effort to produce proof for or against the contention that at least some of the foreign substances inhaled by industrial communities play a part in the origin of the growing numbers of neoplasms.

As a result of this investigation the Laboratory has discovered that a pulmonary adenocarcinoma can be provoked with a high measure of certainty in rats by exposing them for approximately six months to an aerosol of beryllium sulphate. The tumors commence to appear after an induction period of about nine months, and about fourteen months after the onset of the period of exposure there is a sudden progressive peak incidence of carcinomas in various stages of development. There may be as many as three tumors simultaneously in a single animal. This induction period and the total incidence of tumors remain the same no matter whether the beryllium exposure be continued daily for the whole period or whether it be discontinued after an effective dosage has been administered over the first six months.

The cytological components of these tumors betray marked anaplastic phenomena. Metastases have been observed in lymph glands, pleura, kidneys, and other organs studied. On transplantation to the subcutaneous tissues of young rats these lung tumors also survive excellently and continue to proliferate in the manner of true metastases. Two basic types of neoplasms have so far been identified, namely, an alveolar and a squamous cell type.

Further information which is pertinent to this whole question refers to the fact that the beryllium does not undergo any alteration in the lung tissue and may therefore be recovered from the lungs of rats and thus be quantitatively correlated with the occurrence of lung cancer. Furthermore, it has thus far been possible to show that neither quartz nor iron-oxide dust inhalation has any influence in facilitating the onset of the cancers.



i.e. they do not act as co-carcinogens. The role of asbestos in this connection is in the process of being explored.

It is impossible to avoid observing the hue and cry which has been set afoot over the possibility that the inhalation of tobacco smoke may serve as an explanation for the rising incidence of lung cancer. The statistical correlation between smoking habits and lung cancer incidences is no doubt somewhat suggestive and the demonstration that tobacco tars contain carcinogenic fractions compels one to consider the theory seriously. Immediately, the following questions come to mind: Why do these carcinogens only act on certain individuals and particularly on males rather than females? This may not merely be a question of endocrines or intensity or duration of their smoking habits but may well be related to the fact that men are more commonly exposed to environmental respiratory hazards of a variety of kinds. Why also do the carcinogens act more strongly on the lung than on the more proximal portions of the respiratory tract where these potential carcinogens should have the greatest gradient of concentration? What factor determines that a cancer shall arise at any particular locality in the lung? If smoke is the cause, why does it produce a single lesion instead of multiple neoplasms? Having shown that tobacco tar contains carcinogens, has one disproved that other carcinogenic agents derived from the environment, for instance in the air breathed by the smoker or breathed in the process of smoking and altered or concentrated through that process may not be potent factors in the production of these cancers? Our statistics have not adequately differentiated between the incidence of lung cancer in industrial versus non-industrial populations.

A higher probability appears to be that before the potential carcinogenic substances in tobacco smoke can have any specific influence in provoking abnormal cell growth they must be implanted in a ready prepared tissue bed where they would operate synergistically with other substances. Such co-carcinogens may be metabolic products deriving from the host tissue, e.g., endocrine derivatives, or they may have been introduced into the lungs from the environment. It is the current consensus of opinion that the neoplasm phenomenon thus has its origin in multiple causes.

It is now proposed to use the beryllium method in relation to tobacco smoke. By rendering lung tissue cells unstable through prior exposure to beryllium the addition of a second carcinogenic agent ought to precipitate lung tumors at a greater rate and sooner than would at present be anticipated for beryllium alone. It is proposed to use tobacco smoke at the secondary potential co-carcinogen.

While not wishing to deny entirely the merits of experiments which have already been extensively conducted on tumor-susceptible mice, it must be pointed out that when one starts off with an unknown primary carcinogen, or series of carcinogens as in these mice, it becomes extremely difficult to interpret the effect of a substance under test, e.g., tobacco tars, while there is no known method of quantitating these unknown primary co-carcinogens. The beryllium method applied to the rat not only has the advantage

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that the beryllium actually deposited in the rat lung can be quantitatively determined by means of methods perfected in our biochemistry department, but it must also be emphasized that the tumor to be provoked is one which does not occur naturally in these rats so that it is a specific lesion provoked by a known substance and whose statistical incidence bears a calculable relationship to the amount of beryllium retained in the lung tissue of the experimental animal. Provided, therefore, that these factors are properly controlled as they may be, any enhanced or earlier incidence of tumors in animals exposed to tobacco smoke would signify a synergistic relationship between them. Should the tobacco smoke, on the other hand, retard the onset of tumors or diminish the total incidence of these, one might be able to infer an anticarcinogenic effect.

## II. DETAILED PLAN OF PROCEDURE

- A. 250 rats will be exposed to an aerosol comprising a known constant concentration of beryllium sulphate dust introduced into the atmosphere of the experimental chambers for eight hours each day, 5 days per week for a total period of six months. It is anticipated that a number not exceeding about fifty of these rats may succumb to acute beryllium toxicity.
  - (a) Half of the surviving rats will be daily exposed to freshly produced tobacco smoke for an additional period of six months.
  - (b) The remaining half will be transferred to fresh air to serve as a control.
- B. The experimental procedure will be reversed by exposing 400 rats to inhalation of fresh tobacco smoke for six months and thereafter dividing the survivors into three groups, viz:
  - (a) Half of the survivors to undergo exposure to the beryllium sulphate aerosol.
  - (b) A quarter to revert to residence in clean air.
  - (c) The remainder to continue exposure to the smoke chamber.
- C. 50 rats of the same age and strain to be kept in normal air throughout the duration of the study as a further control to A and B.

## III. X-RAY CONTROL

At monthly intervals sample animal batches from each group (except those currently in the beryllium chambers) will be subjected to macro-radiography as the onset of lung tumors can thus be well controlled. This method will determine or modify the fate of individual animals.

## IV. SACRIFICING SCHEDULE

Animals of groups A and B will be sacrificed in sample batches at 3, 6, 9, 10, 11, 12, 13, 14, 15 months after the commencement of exposure to either tobacco smoke or beryllium sulphate dust. Any suspect animals discovered by radiography will be similarly sacrificed. All animals will be subjected to histological study and biochemical assays for beryllium will be run on representative samples and organs.

Proposal - Tobacco Smoke  
T.I.R.C. - 10-1-54

## V. PRECAUTIONS AGAINST BERYLLIUM

Certain human individuals have shown an exaggerated susceptibility to the toxic effects of beryllium compounds. It is therefore necessary to conduct the experiments by means of beryllium with elaborate circumspection. Special equipment and methods have been elaborated in The Saranac Laboratory during the past fourteen years to ensure absolute safety for the personnel and the public. This, however, causes considerable expenses. The Laboratory has the advantage of having a great deal of the basic equipment, including four special chambers, each with the capacity for 100 animals, to house all the experimental animals for the projected experiment. One new cage will have to be constructed, besides special apparatus to create the tobacco smoke.

## VI. BUDGET

### A. Personnel and Salaries

PERSONNEL		SALARIES			
	No.	\$-Hourly	1st Yr.	2nd Yr.	Total
Pathologists	3	4.00	\$1,500.	\$3,000.	\$4,500.
Biochemist	1	3.00	500.	1,000.	1,500.
Chemist	1	3.00	500.	500.	1,000.
Engineer	1	3.00	500.	500.	1,000.
Research Associate	1	3.00	1,000.	1,000.	2,000.
Photographer	1	2.50	500.	500.	1,000.
Histologist	1	2.50	500.	1,000.	1,500.
Technicians:					
Pathology	1	2.00	600.	600.	1,200.
Animal Care	2	2.00	800.	400.	1,200.
Histology	2	2.00	200.	800.	1,000.
Biochemistry	1	2.00	400.	600.	1,000.
Engineering	1	2.00	600.	200.	800.
Mechanic	1	2.00	400.	400.	800.
Typist	1	2.00	200.	500.	700.

Sub-total

\$3,200. \$11,000. \$19,200.

Workmen's Compensation and  
Superannuation Contribution

820. 1,100. 1,920.

Total

\$9,020. \$12,100. \$21,120.

### B. Expendable Supplies (continued on next page)

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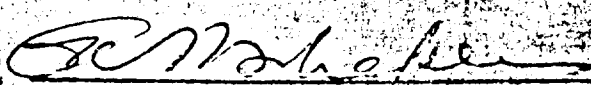
VI. BUDGET (Continued)

A. <u>Personnel and Salaries</u> (Brought forward)	\$21,120.
B. <u>Expendable Supplies</u> (2 years)	
Animals: Rats - 500 at \$2.00	\$1,000.
Maintenance for 5,250 rat/months at \$1.00	5,250.
Insurance against epizootics	2,500.
Experimental Chambers	
Maintenance of 5 chambers at \$1,500. per annum	3,000.
Supplies:	
Histological	475.
Photographic	580.
Radiographic	560.
Chemical (mainly beryllium)	500.
Glassware	100.
Miscellaneous	250.
Materials for adapting dust chambers	250.
	\$14,465.
C. <u>Equipment</u> (Permanent)	
New generator for macro-radiography unit	\$2,000.
Additional new beryllium chamber	1,000.
Animal cages (replacements)	250.
Microphotographic accessories	250.
	3,500.
D. <u>Overhead</u>	
At 25%	9,771.
E. <u>Travel</u>	
Contingent fund	500.
GRAND TOTAL FOR TWO YEARS. . . . .	\$49,356.

Note: (1) Funds requested for the first year \$29,356.  
It would be appreciated if at least \$3,000. could be made available well in advance of any date of commencement preferred by the Committee to enable the construction of special units and the acquisition of special equipment.

(2) The Saranac Laboratory is a non-profit organization incorporated under the State Education Act by the Board of Regents of Albany, New York.

October 1, 1954

  
G. W. H. Schepers, M.D., D.Sc., F.C.C.P.  
Director, The Saranac Laboratory

GWHS:LB

Schour

OUT

~~CROSS-REFERENCE-SHEET~~

Name or Subject

TIRC progress report #1, dated April 18, 1956, TIRC  
grant #84 to Dr. Isaac Schour (University of Illinois);  
subject: "Histologic Alterations in Oral and Nasal Mucosa  
Following Smoking"

Regarding

Sent to Mr. Hewitt on August 18, 1958 - to be returned

SEE

1003541086

Schour

OUT

~~CROSS-REFERENCE-SHEET~~

Name or Subject

TIRC renewal application for research grant -  
Drs. Isaac Schour, Joseph P. Weinmann and Maury  
Massler; subject: "Experimental Studies of the  
Histologic Structures of the Oral and Nasal Tissues  
Exposed to Tobacco Smoke"

Regarding

Sent to Mr. Hewitt on August 18, 1958 - to be returned

SEE

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TOBACCO INDUSTRY RESEARCH COMMITTEE  
150 EAST FORTY SECOND STREET

CF. #2  
NEW YORK 17, N.Y. Activated 1/1/55  
Renewed 1/1/56

Application For Research Grant

Date: March 20, 1958

1. Name of Investigator:

Maurice S. Segal, M.D.

2. Title:

Clinical Professor of Medicine, Tufts University School of Medicine  
Director, Lung Station (Tufts) and Department of Inhalation Therapy, Boston City Hospital

3. Institution

& Address:

Tufts University School of Medicine, 136 Harrison Avenue, Boston, Mass.  
Boston City Hospital, 818 Harrison Avenue, Boston, Mass.

4. Project or Subject:

"Relationship of Cigarette Smoking to Chronic (obstructive) Pulmonary Emphysema"

5. Detailed Plan of Procedure (Use reverse side if additional space is needed):

A number of reports have appeared in the literature suggesting or implying a direct causal relationship between cigarette smoking and the development of chronic pulmonary emphysema. These reports have been based on analyses of very small number of patients. The diagnoses were not always proven, and possible tobacco aggravation of a pre-existing chronic bronchitis may have been confused with the concept of a direct etiologic relationship to chronic pulmonary emphysema.

Lowell, F.C., Franklin, W., Michelson, A.L. & Shiller, I.W. : Chronic Obstructive Pulmonary Emphysema: A disease of Smokers. Annals of Internal Medicine, 45:268, 1956. A study in 34 patients: "The evidence presented indicates that in the New England area and in the age group over 50, smoking is the major cause of emphysema and that the disease is inflammatory rather than degenerative in nature."

I propose to carefully review the records of approximately 300 private patients with proven chronic pulmonary emphysema. Particular attention would be paid to the following:

1. Basis for diagnosis.
2. Patient's personality profile.
- 4.XX Specific tobacco history (amount and duration).
3. Vital statistics of each patient.

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5. Detailed plan of procedure - cont'd.:

5. history of the underlying bronchitis and emphysema.
6. history of associated gastro-intestinal lesions, e.g. ulcer.
7. history of any associated peripheral vascular disease.

A study of this type should reveal statistically significant data and provide a base for further study.

A review of patients through letter A revealed several non-smokers with very severe chronic bronchitis and chronic pulmonary emphysema.

1003541089



6. Budget Plan:

Salaries	secretarial	\$ 3500.00
Expendable Supplies	publications	
Permanent Equipment	present, at meet.	750.00
Overhead (Tufts)	15%	750.00
Other		
Total		\$ 5000.00

7. Anticipated Duration of Work:

Six to twelve months.

8. Facilities and Staff Available:

Private office records and staff.  
Lung Station (Tufts) records and staff.

9. Additional Requirements:

None

10. Additional Information (Including relation of work to other projects and other sources of supply):

None

1003541030

Signature /s./ Maurice S. Segal, M.D.  
Director of Project

/s./ Leonard G. Mead  
Business Officer of the Institution

6. Budget: **TOBACCO INDUSTRY RESEARCH COMMITTEE**  
350 FIFTH AVENUE, NEW YORK 1, N. Y.  
Permanent Equipment  
Application For Research Grant

**EQUIPMENT:**

Other

c. **smoking**

Date:

August 9, 1954

7. Anticipated Duration of Work

1. Name of Investigator: **Maurice S. Segal, M.D.**

2. Title: **Clinical Professor of Medicine, Tufts College Medical School, Boston, Mass.  
Director, Department of Inhalational Therapy, Boston City Hospital.**

3. Institution & Address:

**Tufts College Medical School, 136 Harrison Avenue, Boston, Mass.  
Boston City Hospital, 818 Harrison Avenue, Boston, Mass.  
Department of Inhalational Therapy.**

4. Project or Subject: **SUPPLEMENTARY PROTOCOL FOR STUDY OF THE EFFECTS OF TOBACCO**

9. Additional Acquisition: **SMOKING ON RESPIRATION AND CIRCULATION**

**AIM:** Differentiation between the normal -- tobacco bronchitis and other types of bronchitis.

**DISCUSSION:**

Several communications have appeared in the literature discussing the significance of a type of bronchitis (coughing, wheezing and shortness of breath) (cont'd on back)

5. Detailed Plan of Procedure: (Use reverse side if additional space is needed)

a. **Pre-motachogram:** Study of various air flow rate and volume patterns, and correlation to clinical picture and smoking.

b. **Pneumotachogram:** Study of various air flow rate and volume patterns, and correlation to clinical picture and smoking.

c. **Intrapleural pressure:** Routine intra-esophageal pressure studies but when possible direct intrapleural measurements will be made. Evolution of the visco-elastic properties of the lungs can be made. Differentiation of forces needed to overcome resistance to air flow and resistance to tissue deformation can be made by breathing different gas mixtures on the normal lung to determine the effect on respiration and circulation.

d. **Venous pressure:** Influence of different breathing patterns on circulation and cardiac output.

e. **Arterial pressure:** Influence of different breathing patterns on arterial pressure and cardiac output. Blood gases and pH determinations can be made when advisable.

f. **Measurement of the work of breathing:** The work of breathing may be separated into its different components by plotting of the pressure-volume diagrams obtained from Normal above. In this manner the work to overcome airway elastic forces, viscous forces and the active work during expiration can be determined. (cont'd on back)

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#### 4. (cont'd.)

breath) noted in cigarette smokers. This has been referred to as smokers' or tobacco bronchitis. Allergic and specific irritant factors have been suggested as responsible. Some investigators empirically suggest that all patients with coughing or wheezing of any etiology omit all smoking. Others urge that deliberate pre-operative consideration of the patient's smoking habits be made in judging the type of anesthesia to be employed and measures to prevent post-operative pulmonary complications, which are said to be higher in these patients. On the other hand it appears to some that the mild bronchitis observed in cigarette smokers is so common as to be normal. Most of this data appear as clinical observations.

CONJECTURE AND CONJECTURE ON THE SUBJECT

The effect of cigarette smoking has not been thoroughly investigated in regard to correlation of the effects on respiration and circulation. This should be done to determine what effects, if any, smoking has on the normal lung and in the patient with established bronchitis. The definition of the so-called "tobacco cough and wheeze" at best remains vague. Differentiation of the various types of effect is important. PROJECT OR SUBJECT IS: EFFECTS OF TOBACCO SMOKING ON RESPIRATION AND CIRCULATION

Study of normal non-smokers of various age groups in decades.

A. Study of normal smokers of various age groups in decades.

a. Cigarette smokers with and without habitual inhalation.

b. Cigar smokers with and without habitual inhalation.

c. Pipe smokers with and without habitual inhalation.

3. Study of smokers with tobacco bronchitis in relation to age, amount of smoking and habitual inhalation.

of patients

a. Cigarette smokers.

b. Cigar smokers.

c. Pipe smokers.

4. Study of bronchitis of other types in:

a. Non-smokers.

b. Smokers.

c. Smokers with tobacco bronchitis.

5. (cont'd.)

6. Correlation of the above data with:

- Routine pulmonary function studies — lung volumes and subdivisions, maximum breathing capacity, index of intrapulmonary mixing, and arterial blood gas studies.
- Clinical and x-ray picture.
- Smoking.

#### EQUIPMENT:

Complete pulmonary function study equipment.  
Sanborn 4-channel oscillograph — direct writer.  
Pneumotachograph apparatus.  
Necessary gauges and amplifiers for simultaneous recording.

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6. Budget Plan:

Salaries

Expendable Supplies

Permanent Equipment

Overhead

Other

Total

7. Anticipated Duration of Work:

1. Period of Study

8. Facilities and Staff Available:

Principal Professor of Medicine, Tufts College Medical School, Boston, Mass.  
Director, Department of Inhalational Therapy, Boston City Hospital.

Tufts College Medical School, 136 Harrison Avenue, Boston, Mass.  
Boston City Hospital, 313 Harrison Avenue, Boston, Mass.  
Department of Inhalational Therapy.

9. Additional Requirements: **FLUENT VENTILATION FOR STUDY OF THE EFFECTS OF TOBACCO  
SMOKE ON EXPIRATION AND CIRCULATION**

1. Effect of tobacco smoke on the normal -- tobacco bronchitis and other types of bronchitis.

2. Effect of tobacco smoke on the circulation.

Several observations have appeared in the literature discussing the

10. Additional Information (Including relation of work to other projects and other sources of supply) **cont'd. as back**

1. **Experimental Design:** Study of various air flow rates and volume patterns, and correlation to clinical practice and findings.

2. **Experimental Design:** Routine intratracheal pressure studies but when possible direct intratracheal measurements will be made. Evaluation of the various patterns of breathing of the subject under study. Differentiation of, for example, normal breathing from obstructive breathing and the effects of various types of breathing on the circulation.

3. **Experimental Design:** Influence of different breathing patterns on circulation and cardiac output.

4. **Experimental Design:** Influence of different breathing patterns on arterial pressure and cardiac output. Blood gases and pH determinations can be made under conditions of different breathing patterns.

5. **Experimental Design:** The value of breathing apparatus.

6. **Experimental Design:** The value of breathing apparatus. The value of breathing apparatus in the treatment of various types of breathing disorders. The value of breathing apparatus in the treatment of various types of breathing disorders. The value of breathing apparatus in the treatment of various types of breathing disorders.

Business Officer of the Institution

1003541093

## ROUTING SLIP

NO. 618From: ENDDate July 18To: AECK - Comment and returnTIRC

Please initial, date and forward

Origin: R. C. Hockett with TIRC 7-14-55Re: Work of Dr. Segal on breathing mechanics

- |  |  |
|--|--|
| <input type="checkbox"/> Answer Direct   | <input checked="" type="checkbox"/> Approve and return |
| <input type="checkbox"/> Draft reply     | <input checked="" type="checkbox"/> Comment and return |
| <input type="checkbox"/> Discuss         | <input type="checkbox"/> For your information          |
| <input type="checkbox"/> Note and return | <input type="checkbox"/> Please see me                 |
|  | <input type="checkbox"/> Note and file                 |

Remarks: I plan to visit him Tues. Jul. 26, prepared to  
describe our work + see his ~~transcript~~ ~~write~~ ~~see~~ ~~etc~~ ~~etc~~  
7/27 - ~~He~~ I dict. a memo re visit of 7/26. Reply about 8/1.  
Trip worthwhile - eqpt possibly useful. GRC

1003541094

TOBACCO INDUSTRY RESEARCH COMMITTEE

5320 EMPIRE STATE BUILDING  
NEW YORK 1, N. Y.

July 14, 1955

Dr. Robert N. DuPuis  
Vice President - Research  
Philip Morris, Inc.  
Box 1859  
Richmond, Virginia

Dear Bob:

I received your letter of July 5th, which commented upon the work of Dr. Segal on breathing mechanics. When the letter arrived I was just preparing to hop off for Boston where I expected to see him.

I did see him on Tuesday afternoon and had a very good talk about the work in progress. He was much interested in the measurements on smoking mechanics and I could see that my account of the efforts being made in this direction stimulated his thinking and his interest.

I feel sure that you and he would both benefit from a conference at first hand. He would be very glad to have you visit the laboratory and to show you all the mechanical devices in use there. This would be far more effective than any attempt to transmit a description of instruments and methodology by letter.

Do you think you could arrange to go on up there sometime when you are in New York? I will be glad to act as an intermediary in any way that will help.

If you cannot make a visit any time soon, I will try to get written descriptions for you, but I strongly recommend a first-hand conference.

Very sincerely,

Bob

Robert C. Hockett  
Associate Scientific Director

RCH:etk

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RECEIVED  
JUL 1 1961

RECEIVED  
JUL 1 1961  
PHILIP HARRIS & CO. LTD. INC.  
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1003541096

July 5, 1955

Mr. Robert C. Hockett  
Associate Scientific Director  
Tobacco Industry Research Committee  
5320 Empire State Building  
New York, New York

Dear Bob:

As you know, we are attempting a small-scale study of the mechanics of human smoking. We have assembled some crude instruments for this work but are constantly on the lookout for possible improvements.

The first Project Report of the Study of Effects of Smoking on Normal Subjects and Patients with Pulmonary Disease refers briefly to certain instruments and techniques used for recording instantaneous air flow rates and integration thereof to permit direct recording of air volume flows.

Would it be possible for us to obtain from Dr. Segal through you a more detailed description of his instruments and techniques in order that we might determine whether they would be applicable in our problem? I feel that the matter of human smoking mechanics is of extreme importance and that cooperation of various laboratories in this effort would be highly desirable. As an example, unless we know the puff volume, smoke composition and volume of inhaled air, we cannot arrive at an estimate of the concentration of smoke constituents in the lung and therefore cannot estimate possible chemical effects on a national basis.

Sincerely,

Robert N. DuPuis  
Vice President - Research

RND:BS

1003541097



THE BOSTON CITY HOSPITAL  
818 Harrison Avenue, Boston 18, Mass.

DEPARTMENT OF INHALATIONAL THERAPY AND  
LUNG STATION (TUFTS)  
MAURICE S. SEGAL, M.D., DIRECTOR

June 14, 1955

## FIRST PROGRESS REPORT OF THE TOBACCO INDUSTRY RESEARCH COMMITTEE

## GRANT-IN-AID STUDY OF

"THE EFFECTS OF CIGARETTE SMOKING ON NORMAL SUBJECTS AND PATIENTS  
WITH PULMONARY DISEASE"

- able for W.F.'s work?*
1. Our initial efforts have been spent in more fully equipping the laboratory so that the more important aspects of the Mechanics of Breathing could be studied. A used but adequate fluoroscopic unit, two additional transducers for instantaneous air flow rate studies (pneumotachography) were obtained and an electrical unit to permit direct recording of the air volume flows by integration from the pneumotachograph tracings was constructed. At the present time we are attempting to actually record the intensity of cough on a plastic belt play-back recording unit (loaned to us for this purpose) and to simultaneously integrate these frequency responses with our recorded intraesophageal pressures, instantaneous air flow rates, volume rates and venous and arterial pressures. This would permit an objective physiologic correlation of the effects on the cardiopulmonary apparatus of both spontaneous and induced cough in both the normal subject and the patient with pulmonary disease. It should permit an accurate physiologic analysis of the so-called "tobacco cough" and the "bronchitic cough," which we need for the Second Phase of our planned studies.
  2. The controls necessary for a study involving the complicated factors in the Mechanics of Breathing were first evaluated. The effects of various positions and the degree of distention of the esophageal balloons employed in our pressure-volume studies had to be determined, before the various factors involved in the mechanics of breathing could be evaluated. The mechanics of breathing were then studied in 11 normal subjects and 27 patients with pulmonary disease. In the normal subjects there were: One non-smoker; six moderate smokers (up to 1 package daily); and four heavy smokers (more than one package daily). In the patient group there were three non-smokers; 18 moderate smokers and six heavy smokers. Three patients were studied on two or more occasions and two patients were studied after a period of several weeks of abstinence from smoking.
  3. It was then considered advisable to evaluate the Positional Factors involved in the mechanics of breathing. Seven normal subjects and seventeen patients with pulmonary disease were studied in different body positions. This data is presently being correlated and will be the subject of at least two basic papers in pulmonary physiology. From these studies it becomes essential that the effect of body positions be considered in all studies involving the effects of drugs and therapeutic agents on the mechanics of breathing. Comparison studies of these effects have little value unless the physiologic effects of body positions alone are understood and taken into consideration.

1003541098

June 14, 1955

4. Acute studies have been started on the effects of smoking one or two cigarettes on the mechanics of breathing. One normal subject and four patients with pulmonary disease have been fully studied. One patient showed a decrease in compliance (increased stiffness of the lung) during quiet breathing with an accentuation of this drop during rapid breathing and an increase in mechanical resistance. In the other four subjects the mechanics of breathing were improved after smoking during normal breathing, timed vital capacity efforts and during cough ("exhorted").

These numbers are too small for any conclusions and our studies along these lines are continuing.

/s/ Maurice S. Segal, M.D.

Maurice S. Segal, M.D.  
Director, Lung Station (Tufts) and  
Department of Inhalational Therapy  
Boston City Hospital

Ernest O. Attinger, M.D.  
Research Fellow in Medicine, Tufts College  
Medical School  
Senior Resident, Department of Inhalational Therapy, Boston City Hospital

1003541039

## TOBACCO INDUSTRY RESEARCH COMMITTEE

6. Budget Plan for 1956

350 FIFTH AVENUE

NEW YORK 1, N. Y.

**RENEWAL**

Salaries  
Expendable Supplies  
Application For Research Grant  
Overhead  
Other

20%  
5%  
30%  
10%  
5%

#2 R1

Date: September 28, 1955

OK

1. Name of Investigator: Maurice S. Segal, M.D.  
Ernst O. Attinger, M.D.

2. Title: Dr. Segal: Clinical Professor of Medicine, Tufts University School of Medicine,  
Director, Lung Station (Tufts) and Dept. of Inhalation Therapy,  
Boston City Hospital

3. Institution: Dr. Attinger: Research Fellow in Medicine, Tufts Univ. School of Medicine  
& Address: Tufts University School of Medicine, 136 Harrison Ave., Boston, Mass.  
Boston City Hospital, 818 Harrison Ave., Boston, Mass. President in  
Residence, Dept. Lung Station (Tufts) and Dept. of Inhalation Therapy

4. Project or Subject: The Effects of Cigarette Smoking on Normal Subjects

5. Additional Requirements: and Patients with Pulmonary Disease.

This report is available within 4 months and will be available in the form of a book of 20,000 pages of H<sub>2</sub>O, O<sub>2</sub>, and CO<sub>2</sub> in respiratory gases.

2. "Respiratory Analyzer" apparatus for determination of partial pressures of O<sub>2</sub> in arterial and mixed venous blood. This instrument is currently in process of construction and will probably be ready for experimental use within 6 months. (Instrument Co., Stamford, Conn.) Its available price is \$1,000.

3. 5. Detailed Plan of Procedure (Use reverse side if additional space is needed): A large number of subjects (especially normals) need to be studied in the manner outlined in the Second Progress Report, (Sept. 1, 1955), in order to determine the nature of the basic changes in respiration and circulation in the lungs with smoking. Some of the factors involved in the changes of compliance and mechanical resistance are the following: change in hydrostatic pressure and changes in intrathoracic blood volume; changes in midposition with change in relative position and dimensions of the tracheobronchial tree, changes in unequal ventilation; changes in surface tension of the lung; and changes in ventilation-perfusion relationships.

To investigate the above which appear to be responsible for the changes noted in the Second Progress Report, the following studies should be carried out in addition to continuing our present studies:

A. Effect of Nicotine by Injection. This study would give some clues to the question if the effects of tobacco upon the mechanics of breathing is a local or a systemic effect.

B. Effect of Nicotine-Free Smoke. This study would help to differentiate between the nicotine effect and the effect of other substances in tobacco upon the mechanics of breathing. *prob. can't elim. nicotine only. describe ion exch. work*

C. Effect of Tobacco on Pulmonary Circulation. (Catheterization Studies).  
A number of investigators have stated that tobacco has an insidious influence on the arteries and on the heart, with a predominant effect on the coronary circulation.

Signature of Project Director  
Business Office of the Institution

1003541100

Tachycardia, rise in blood pressure and vasoconstriction in the skin have been provoked by the smoking of one cigarette. All three phenomena are usually explained by the nicotine stimulation of the sympathetic ganglia, stimulation of the vasoconstrictor center and the stimulation of the adrenals. The most characteristic quality of nicotine is its influence on the automatic ganglia, which after a short period of stimulation become partially blocked or paralyzed. There is considerable doubt if the pulmonary circulation is subject to reflex mechanism and to the action of drugs which influence peripheral circulation. As the mechanics of breathing are also influenced by the pulmonary blood flow and the intrathoracic blood volume, it seems imperative to study ventilation and circulation at the same time. In order to assure an efficient lung function, an effective gas exchange has to be achieved by a minimal work of breathing and work of the right ventricle. Ventilation might very well improve, while circulation and gas exchange gets worse and vice versa. Therefore catheterization studies including pressure measurements of right auricle, right ventricle and pulmonary pressures, as well as the determination of cardiac output, would be indicated. Furthermore these experiments might shed some light upon the presence or absence of reflexes governing pulmonary circulation.

Our experiments would be conducted as follows in a series of (A) normal subjects and (B) patients. Ventilatory mechanics and hemodynamics would be measured simultaneously under:

- a. Normal breathing.
- b. Induced hypoxia (breathing 14 per cent oxygen)
- c. After smoking one cigarette

D. Effect of unequal ventilation as studied by Rawlert Fowler's single breath technique and by analysis of nitrogen elimination rates over longer periods of time. *injected, I presume. What is previous*  
Decrease of compliance with respiratory rate is usually explained on the basis of unequal ventilation. We have certain experimental data, suggesting that this is not *un-esp. immediately* the only factor, but that a change in surface tension brought about by change in *smoking history* intrathoracic blood volume and secretions might also be responsible.

The determination of nitrogen clearance curves by a mass spectrometer offers a relatively simple method to judge the evenness of ventilation at different respiratory rates, and these experiments are of particular interest in view of our findings of the change of compliance with change in respiratory rate in patients with pulmonary disease.

1003541101

6. Budget Plan: for 1956

TOBACCO INDUSTRY

350 FIFTH AVENUE

RENEWAL	Salaries	50%	\$11,000.00
	Expendable Supplies	5%	1,100.00
	Permanent Equipment	30%	6,600.00
	Overhead	10%	1,100.00 2,200.00
	Other	5%	1,100.00
Date: Sept. 1, 1955			Total \$22,000.00

7. Anticipated Duration of Work: on M. Segal, M.D.  
Two Years

8. Facilities and Staff Available:

Facilities: Dept. of Inhalation Therapy and Lung Station (Tufts) Boston City Hospital; Laboratory and Clinic Facilities, Boston City Hospital; Clinical Facilities

Staff: Maurice S. Segal, M.D., Director, Lung Station (Tufts) and Clinical Professor of Medicine, Tufts University School of Medicine. Ernst O. Attinger, M.D., Research Fellow in Medicine, Tufts University School of Medicine. Dr. Merrill Goldstein, Resident in Medicine, Dept. of Inhalation Therapy, Boston City Hospital. Mrs. D. Belgard, Chief Technician--Scholander, Pulmonary Function Studies, etc. Miss T. Adelman, Assistant Technician--Van Slyke and Pulmonary Function Studies, etc.

9. Additional Requirements:

1. Mass spectrometer (Beckman Model 150), approximately \$5,000. This could be available within 4 months and would be used for continuous analysis of  $N_2$ ,  $O_2$  and  $CO_2$  in respiratory gases.

2. "Haemoxymeter" apparatus for determination of partial oxygen tension in arterial and mixed venous blood. This instrument is actually in process of production and will probably be ready for experimental use within some months (Liston-Becker Instrument Co., Stamford, Conn.) No available price as yet.

3. 10. Additional information (including relation of work to other projects and other sources of supply) for at least one ECG machine, \$800.

10. Additional information (including relation of work to other projects and other sources of supply) for at least one ECG machine, \$800. This project is maintained as entirely independent. However, supplemental salary assistance to the technicians and aid for the Assistant Resident in Medicine is supplied through the cooperation of the Trustees of Tufts University School of Medicine and the Boston City Hospital.

For further information, please see Second Progress Report of September 1, 1955.

To investigate the above which appear to be responsible for the changes state in the Second Progress Report, the following studies should be carried out in addition to continuing our present activities:

A. Effect of Nicotine on Breathing. This study would give some insight into the question of the effects of tobacco upon the mechanics of breathing is a local or a systemic effect.

B. Effect of Nicotine on the State. This study would help to differentiate between the nicotine effect and the effects of other substances in tobacco upon the mechanics of breathing.

Signature: M. S. Segal, M.D.

Director of Project

C. Effect of Tobacco on Pulmonary Circulation. (Characterization Studies). Several investigators have stated that tobacco has an insidious influence on the circulation and on the heart, with a predominant effect on the coronary circulation.

J. M. Hayman, Jr.  
Business Officer of the Institution

TOBACCO INDUSTRY RESEARCH COMMITTEE

TUFTS UNIVERSITY  
School of Medicine

Maurice S. Segal, M.D., Director  
Lung Station (Tufts)  
Department of Inhalation Therapy

Boston City Hospital  
818 Harrison Avenue  
Boston 18, Massachusetts

4 March 1959

Robert C. Hockett, Ph.D.  
Tobacco Industry Research Committee  
150 East Forty-Second Street  
New York 17, New York

Dear Dr. Hockett:

Would you please forgive my delay in answering your letter for I have been away a good deal this past month.

The studies are progressing slowly but I believe will be rewarding. In addition to the survey I am making on the private-type patient, in which I am particularly concerned with, if any, the inter-relationship of smoking habits, ulcer and pulmonary emphysema, I have extended the studies into the medical hospital population. Dr. Sanford Chodosh, our American Trudeau Fellow and Dr. John Simpson, a resident at the Boston City Hospital, are carrying out exhaustive questionnaire and laboratory studies in the hospital patients with chronic pulmonary disease as well as a control group with non-chronic pulmonary disease. The questionnaire and routine to which these patients are subjected to is enclosed. Dr. Chodosh has drawn up a summary of the first six months' study. I believe his preliminary observations are quite revealing and in fact appear to be running very close to the observations that I have been making in the first mentioned group of patients. It certainly is too early to draw any conclusions. At the present time, it does not appear likely that peptic ulcer or pulmonary disease are exclusively diseases of the heavy smoker.

I am afraid that with the extension of our most ambitious survey study that we will probably run on for another year before attempting any statistical analyses. I do hope you will be patient with us, for I sincerely believe this will be a very valid study.

With all best personal wishes, I am,

Sincerely yours,

/s/ Maurice S. Segal, M.D.

Maurice S. Segal, M.D.  
Director

MSS:BAW  
enc.

1003541103

CONFIDENTIAL

TIRC Grant #2R1 & 198

Report No. 4

Maurice S. Segal, M.D.  
Tufts University School of Medicine

March 2, 1959

Effects of cigarette smoking in normal subjects on pulmonary function.

Relationship of cigarette smoking to chronic (obstructive) pulmonary emphysema.

This study was primarily designed to determine the true incidence of peptic ulcer disease in patients with chronic pulmonary disease. Consecutive patients with chronic lung disease on the medical wards of the Boston City Hospital were evaluated by history, physical examination, pertinent laboratory tests and upper gastrointestinal roentgen studies. Controls were similarly evaluated. They were also chosen from the ward patients and matched for age, sex and relative degree of chronic disease.

The study is presently less than a third completed. Twenty-five pulmonary patients have been studied. Twenty per cent had evidence of peptic ulcer by x-ray. Three had gastric ulcers and two had chronic duodenal ulcers. Three of these patients were non-smokers. Of the other twenty patients, at least seven had mild to moderate symptoms suggestive of peptic ulcer disease but did not have ulcers demonstrated on gastrointestinal series.

Ten patients were evaluated in the control group. Of these, two or possibly three had peptic ulcer disease.

It is obviously too soon to draw conclusions of any consequence since the number evaluated is not significant. Of interest, is the fact that 3 per cent of the five ulcer patients with pulmonary disease were non-smokers and two of the five had only mild pulmonary disease. Although the number evaluated is small, it is also interesting that approximately the same per cent of controls had peptic ulcer disease as did the group with chronic pulmonary disease.

1003541104

1. NAME

ADM. DATE:

ADDRESS

DISC. DATE:

WARD

HOSPITAL NUMBER

2. DATE OF OBSERVATIONS

OBSERVOR'S NAME

3. AGE

4. SEX

5. FAMILY HISTORY:

a) PULMONARY DISEASE

b) GASTRO-DUODENAL DISEASE

6. RESIDENCE HISTORY:

WHERE

WHEN

a)

b)

c)

d)

7. OCCUPATIONAL HISTORY:

a) TYPE OF WORK

b) PHYSICAL CHARACTERISTICS OF PLACE OF WORK:

1. SIZE

2. VENTILATION

3. HUMIDITY

4. FANS

5. BLOWERS

6. TEMP.

7. DUSTS

8. FUMES

9. SMOGS OR FOGS

10. VAPORS

11. MISC.

c) ELEMENTS EXPOSED TO:

d) INDUSTRIAL PRECAUTIONS

e) FREQUENCY OF X-RAYS:

f) X-RAY THERAPY:

g) RELATIONSHIP OF SYMPTOMS TO WORK:

8. SOCIO-ECONOMIC FACTORS:

a) INCOME

b) ABILITY TO LIVE WITHIN MEANS

c) MISC.

1003541105



## 9. HABITS

## a) SMOKING:

AMOUNT/DAY (NOTE CHANGES)DURATION OF USE

1. CIGARETTES
2. CIGARS
3. PIPES
4. CHEWING TOBACCO

RELATIONSHIP OF SYMPTOMS TO SMOKING:

INHALATION OF SMOKE:

## b) ALCOHOL:

1. TYPE

2. AMOUNT

3. DURATION

## c) DRUGS:

NAMEDOSAGEDURATIONSIDE EFFECTS

1.

2.

3.

4.

5.

## d) THERAPEUTIC RESPONSE TO:

1. BRONCHODILATORS
2. AMINOPHYLLIN
3. STEROIDS
4. ANTIBIOTICS
5. STOP SMOKING
6. MISC.

## 10. PAST HISTORY:

WHENSPECIAL SYMPTOMSRx & SEQUELAE

- a) PERTUSSIS
- b) MEASLES
- c) SINUSITIS
- d) TUBERCULOSIS
- e) INFLUENZA

1003541106

f) NOXIOUS INHALANTS

g) PNEUMONIA (TYPE)

h) COMMON COLDS

i) BRONCHITIS

j) ALLERGY

1. NASAL POLYPI

2. PERENNIAL VASOMOTOR RHINITIS

3. SEASONAL HAYFEVER

4. URTICARIA

5. ECZEMA

6. DRUG REACTIONS

ASPIRIN

PENICILLIN

COCAINE

MORPHINE

OTHERS

7. SKIN TESTS

8. HYPOSENSITIZATION THERAPY

k) BRONCHIAL ASTHMA

1. ACUTE

2. CHRONIC

3. STATUS

4. SEASONAL

5. PERENNIAL

6. ASSOCIATED WITH OTHER ALLERGIES

7. FAMILIAL ALLERGIES?

l) COUGH

1. DRY

2. PRODUCTIVE

3. PAROXYSMAL

1003541107

4. CROUPY

5. TIME OF DAY

m) SPUTUM

1. DIURNAL PRODUCTION

2. SEASONAL PRODUCTION

3. CHARACTER - COLOR:

CONSISTENCY:

4. HEMOPTYSIS

5. AMOUNT

6. CHANGE OVER THE YEARS

n) SHORTNESS OF BREATH:

1. AT REST

2. EFFORT

3. ORTHOPNEA

4. # PILLOWS REQUIRED

5. ANY CHANGES

o) PREVIOUS PULMONARY DIAGNOSIS -

      GROUNDS ON WHICH IT WAS BASED -

p) PREVIOUS PULMONARY FUNCTION STUDIES -

      RESULTS?

q) PREVIOUS CHEST X-RAYS

r) PREVIOUS SINUS X-RAYS

11. PAST HISTORY - GASTROINTESTINAL

a) PREVIOUS Dx OF ULCER

      MEANS OF Dx

FREQUENCY

DURATION

TYPE OF SYMPTOMS

b) INDIGESTION

c) HEARTBURN

d) NAUSEA

e) VOMITING

f) MELENA

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FREQUENCYDURATIONTYPE OF SYMPTOMS

g) FOOD INTOLERANCES

h) GALLBLADDER Sx

i) BOWEL HABITS

j) CHARACTER OF STOOLS

k) USE OF DRUGS FOR INDIGESTION:

1. TYPE

2. DOSAGE

3. FREQUENCY OF USE

l) HEMETEMESIS:                      WHEN                      AMOUNT

m) EPIGASTRIC PAIN

1. CHARACTER

2. DURATION

3. RADIATION

4. FREQUENCY

5. SEASONAL

6. DIURNAL

n) PREVIOUS X-RAYS

o) ASYMPTOMATIC

p) MISCELLANEOUS

IV. PHYSICAL EXAMINATION

a) SINUSES

1. SWELLING, LOCAL

2. TENDERNESS

3. TRANSILLUMINATION

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b) NOSE

1. PALLID
2. BOGGY
3. DRY
4. CYANOTIC (BLUE)
5. RED

## d) NASAL POLYPI

## e) TONSILS

## f) POST NASAL CONTENTS

## g) EARS:

1. WAX?
2. INFLAMMATION?
3. HEARING STATUS

## h) CHEST CONFIGURATION

1. MOVEMENT
2. A-P: TRANSVERSE
3. KYPHOSIS
4. SCOLIOSIS
5. PECTUS EXCAVATUM
6. OTHER

## i) RESPIRATORY RATE

## j) RESPIRATORY CHARACTER

1. SIGHING
2. HYPERVENTILATION
3. SHALLOW
4. THORACIC
5. DIAPHRAGMATIC
6. PURSED LIPS

c) THROAT

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## 7. ACCESSORY MUSCLES

## 8. MISC.

k) TACTILE FREMITUS

l) PERCUSSION

m) DIAPHRAGM POSITION:

L.  
MOVEMENT: R.

n) BREATH SOUNDS

1. GENERAL CHARACTER

2. LOCALIZED DIFFERENCES

o) INSPIRATORY: EXPIRATORY PHASE  
GENERAL

p) BRONCHOSPASM: LOCAL

q) RALES &amp; RHONCHI

r) FRICTION RUBS

s) VOCAL FREMITUS

t) EGOPHONY OR PECTORILOQUE

u) CARDIAC:

1. RATE

2. RHYTHM

3. SIZE - MAX. CM FROM MSL OF DULLNESS <  $\begin{matrix} L \\ R \end{matrix}$ INTERSPACE  
INTERSPACE4.  $P_2 : A_2$  = > <

5. RIGHT VENTRICULAR THRUST

6. CHARACTER OF HEART SOUNDS GOOD MUFFLED INAUDIBLE(?SP)

v) EPIGASTRIC TENDERNESS

w) HEPATOMEGALY

TENDER?

x) VENOUS DISTENTION

1. NECK

2. PERIPHERAL

y) CLUBBING

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## z) CYANOSIS

1. EYES
2. M. M. OF MOUTH
3. LIPS
4. EXTREMITIES

aa) TOBACCO STAINS ON FINGERS

bb) PERIPHERAL EDEMA

cc) MENTAL STATUS

dd) MISCELLANEOUS

V. LABORATORY

## 1. GENERAL

DATE:

- a) HCT
- b) ESR
- c) WBC
- d) DIFF.  $\bar{c}$  RBC DESCRIPTION
- e) Q'JANT. EOSIN. COUNT  
(FASTING)
- f) STOOLS ( $\times 3$  min.)
- g) ELECTROLYTES:
  1. CO<sub>2</sub>
  2. Cl
  3. Na
  4. K
  5. OTHER
- h) RECTIC COUNT

## 2. X-RAYS:

- a) CHEST: PA - INSP. & EXP.
- $\bar{c}$
- LATERAL;

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6' HEART FILM

RAO

LORDOTIC

b) GI SERIES:

c) SINUS SERIES:

3. EKG:

4. SPECIAL STUDIES:

	<u>AMT.</u>	<u>EXP.</u>	<u>PER CENT</u>
ROUTINE:			
a) VITAL CAPACITY			
b) MAXIMAL BREATHING CAP.			
c) BREATH HOLDING TIME			
d) CHEST EXPANSION:	EXPIRATION:	INSPIRATION:	
SPECIAL:			
e) RESTING MINUTE VENT.			
f) N <sub>2</sub> WASH OUT			
g) RESIDUAL VOLUME			
h) ALVEOLAR CO <sub>2</sub>			
i) ARTERIAL O <sub>2</sub>			
j) " CO <sub>2</sub>			
k) " pH			
l) MAXIMAL HISTAMINE STIMULATION			
m) SPUTUM & BLOOD SMEARS			

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NOTES: - PROGRESS AND MISC:

CONDITION AT DISCHARGE:

DISCHARGE Dx:

1. PULMONARY
2. GASTROINTESTINAL
3. OTHER

AUTOPSY?

WHEN

NUMBER

1003541114

CHRONIC PULMONARY GROUP - HOSPITAL PATIENTS

PT.	AGE	SEX	SMOKING	PULMONARY DIAGNOSIS	SEVERITY OF PULMONARY	X-ray	ULCER		Oper.	COMMENTS
					DISEASE		History	Previous Diagnosis		
D.M.	66	M	Mod.	C.B. Emphysema. Rt. Intra- thoracic kidney.	Mild	Neg.		At age of 63.	At age of 63.	
G.T.	68	M	Heavy	CPE, CB & Fibrosis. ? Br'ect.	Sev.	Neg.	Previous not now.			
J.H.	63	M	Mod.	CB, Br'ect. Pulm. embo- lus.	Mild	Neg.				
J.Mc.	59	M	Heavy	CPE, CB & Br'ect. Old Tbc.	Mod. Sev.	Chronic duodenal deformity		At age of 38.		Duodenal ulcer chronic, in- active.
W.M.	55	M	Heavy	CPE, CB & Br'ect Fibrosis	Sev.	Neg.	Mild*			Antral gastritis & duodenitis.
P.O.	73	M	Mod. Heavy	CPE, CB & Br'ect. Tbc.-old Cor Pulmonale	Sev.	Neg.	Mod.*			
M.S.	71	F	None	CB RLL pneum.	Mild	Neg.	Mod.			
J.B.	62	M	Mod.	CPE, Old Tbc. ? bysinosis	Mod. Sev.	Neg.	Neg.			
V.C.	64	M	None	CB CPE	Mild	Gastric ulcer	Mod. Sev.			Gastric ulcer, active.

100354115

PT.	AGE	SEX	SMOKING	PULMONARY DIAGNOSIS	SEVERITY OF PULMONARY	ULCER		Previous Diagnosis	Oper.	COMMENTS
					DISEASE	X-ray	History			
M.N.	64	F	Mod.	CB,mild RLL pneum.	Mild	?gastric	Mild*			?gastric ulcer. Hiatus hernia. Diverticulosis of sigmoid.
H.K.	38	F	Very Mild	CB	Mild	Neg.	Mod.*			G.I.Sx.- unexplained.
M.K.	85	F	None	Pulm.Fibrosis & CPE Old Tbc.	Mild	Neg.	Neg.			
J.R.	62	F	None	BA CPE	Mod. Sev.	Neg.	Mod.	Nervous stomach		Hypertension
P.J.	13	M	Very mild	CB	Mild	Neg.	Neg.			
M.Mc.	50	F	Very mild	CB-mild Kyphosis	Very mild	Neg.	Neg.			Ulcerative colitis
C.F.	43	F	None	CB CPE Metastatic CA	Mild to Mod.	Neg.	Slight			Lupus erythemato- sis. CA of breast. Pyelonephritis, etc.
G.L.	80	M	Mod.	CPE CB	Mod. Sev.	Neg.	Neg.			
M.K.	44	M	Mod.	CB & Br'ect. ? Sarcoid.	Mod. Sev.	Neg.				
M.P.	70	F	None	CB & Br'ect. Status post LL lobec- tomy 8 yrs. ago	Mod. Sev.	Old Duodenal ulcer.	Mild			Mycotic pharyn- gitis. Chronic duodenal ulcer.

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PT.	AGE	SEX	SMOKING	PULMONARY DIAGNOSIS	SEVERITY OF PULMONARY	X-ray	ULCER		Oper.	COMMENTS
					DISEASE		History	Previous Diagnosis		
M.D.	61	F	None	CPE Tbc, inactive	Mild	Neg.	Slight			Wt. loss-etiol.?
B.D.	67	F	Mod.	CB CPE Tbc, old, inactive.	Mild to Mod.	Neg.	Old G.B. Slight			Wt. loss-etio.?
F.F.	51	F	None	CBA ? cor pulm.	Mod. Sev.	Gastric ulcer	Mod. Sev.			On steroids.
P.B.	56	M	Mod.	CB	Very mild	?neg.	Neg.			Laennec's cirrhosis. Cholelithiasis
S.G.	75	M	Very mild	CPE CBA	Mod. Sev.	Neg.	Neg.			Bell's palsy
W.R.	74	M	None	Chronic pulm. edema	Mod.	Neg.	Mild			Arteriosclerotic congestive heart disease. Hypertensive cardiovascular disease.

\* Upper Gastro-intestinal symptoms of undetermined origin.

Mod. - moderate  
 Sev. - severe  
 Neg. - negative  
 CB - chronic bronchitis  
 CPE - chronic pulmonary emphysema  
 Br'ect. - bronchiectasis  
 Pulm. - pulmonary  
 Pneum. - pneumonia

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## CONTROL GROUP - HOSPITAL PATIENTS

PT.	AGE	SEX	SMOKING	PULMONARY DIAGNOSIS	SEVERITY OF PULMONARY DISEASE	ULCER			COMMENTS & CHIEF DX.
						X-ray	History	Previous Dx. Oper.	
D.L.	51	M	Heavy			Pre- pyloric ulcer	Mod.	By X-ray at age of 45.	Gastric ulcer. Essential hyper- tension.
R.F.	77	M	Slight (Pipe)			Neg.	Neg.	Neg.	?Laennec's cirrhosis. Malnutrition.
E.Mc.	76	M	None			Prob. post- bulbar ulcer	Neg.	Neg.	Barbiturate overdosage.
F.D.	55	M	Heavy			Neg.	Neg.		Arterio- sclerotic heart dis.
R.H.	43	M	Heavy	Lobar pneum.- right upper lobe.		Neg.	Neg.		Heart disease
J.K.	68	M	Mild			?Antral gastritis ? tumor	Mod. Sev.		? antral gas- tritis. ? CA of stom- ach.
G.Mc.	47	F	Mod.			Neg.	Mild		Wt.loss-etiol.? ?hyperthyroid- ism.
M.W.	46	F	None			Neg.	Gall bladder Sx.		Benign nephro- sclerosis & hyper- tension
J.D.	71	M	Mod.	CPE? - no Sx.		Neg.	Neg.		Prostatic hyper- trophy
A.C.	71	F	None			Neg.	Slight*		Digitallis toxicity.

\* Upper Gastro-intestinal symptoms of undetermined origin.

Mod. - Moderate

Sev. - Severe

Neg. - Negative

1003541118

TOBACCO INDUSTRY RESEARCH COMMITTEE  
150 EAST FORTY SECOND STREET NEW YORK 17, N. Y.

Application For Research Grant

Date: October 7, 1958

1. Name of Investigator: Carl C. Seltzer, Ph.D.
2. Title: Research Fellow in Physical Anthropology
3. Institution  
& Address: Harvard University  
Peabody Museum  
Cambridge, Mass.
4. Project or Subject: Morphology and Smoking in College Graduates: A Fifteen-Year  
Follow-up Study.

5. Detailed Plan of Procedure (Use reverse side if additional space is needed):

The purpose of this study is to explore the extent of relationship between objective measures of body build and smoking and smoking patterns in a group of male college graduates who have been part of a fifteen-year follow-up study.

The raw data necessary for this study are available in our files of the Study of Adult Development (Grant Study), Harvard University. As one of the original members of this Study, all the morphological data on the group of subjects were collected personally by the petitioner of this grant and consist of an extensive series of anthropometric measurements, indices, morphological observations, disproportions, and somatotype classifications. The smoking data on the series of 252 college graduates have already been elaborated by other members of the staff of the Grant Study (see studies of Heath, Clark W. and McArthur, Charles, TIRC 1957 Report).

The essence of the project will concern itself with a detailed statistical analysis and evaluation of the relationship between the anthropometric measurements of body build and body build types with smoking and smoking patterns of the 252 subjects. A report embodying the results of this analysis will be prepared in a form suitable for publication.

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The analysis of the data is not to be limited to a general canvasserie of correlative variables. On the contrary, specific areas will be explored based on the investigator's own experience and the results of previous related studies. For example, McArthur has suggested the relationship of smoking patterns to certain personality variables. Since my own studies have indicated relationship between body disproportions and personality traits and behavior, then it would appear consistent to explore the relationship of these disproportions with smoking habits. Similarly, other theories to be tested will include such morphological variables as somatotype, gynandromorphy, etc. In addition, attention will also be directed to the more complex criteria of smoking involving longitudinal smoking patterns and the like.

It is expected that this analysis will indicate, for these data, whether or not the morphological constitution of the individual is associated with smoking and smoking patterns, the particular physical characteristics (if any) which best express such an association, and the most profitable areas in which further morphological research may be pursued.

1003541120

6. Budget Plan:

principal investigator

Salaries 1/5 time  
Expendable Supplies (file)  
Permanent Equipment  
Overhead 121/23  
Other

2500

50

50

555

1800

4955

Total

statistical and clerical time

7. Anticipated Duration of Work:

One year

8. Facilities and Staff Available:

The statistical laboratory of the Department of Anthropology located in the Peabody Museum is available for the mechanics of statistical analysis.

The files of the Study of Adult Development, previous tabulations of the Study data will be made available.

9. Additional Requirements:

10. Additional Information (Including relation of work to other projects and other sources of supply):

Signature /s/ Carl C. Seltzer  
Director of Project

? illegible  
Business Officer of the Institution

Dean, Faculty of Arts and Sciences

Harvard University

1003541121



*Wilson, Chas.  
Little  
Cutler*

TOBACCO INDUSTRY RESEARCH COMMITTEE

150 East Forty Second Street

New York 17, N.Y.

*#262  
(cf. #214  
Activated  
2/15/59)*

Application For Research Grant

Date: December 3, 1959

1. Name of Investigator: Carl C. Seltzer, Ph.D.  
(Co-investigator - Dr. Caroline Bedell Thomas, Associate Prof. of Medicine, The Johns Hopkins University School of Medicine.)
2. Title: Research Fellow in Anthropology, Peabody Museum
3. Institution & Address: Harvard University  
Cambridge 38, Mass.
4. Project or Subject: Harvard--Johns Hopkins Study of Body Form with Smoking, Precursors of Hypertension and Coronary Artery Disease.

5. Detailed Plan of Procedure: The purpose of the initial part of this project is to obtain anthropometric data including standard somatotype photographs on a series of medical students who are the subject of intensive investigation by the Precursors Study of Hypertension and Coronary Artery Disease of the Johns Hopkins University School of Medicine.

The availability of this morphological data will make it possible to explore the extent of relationship between the morphological constitution of the individual and a series of variables including smoking habits, circulatory response to smoking, cholesterol levels, and other observations already obtained in the precursor study.

Anthropometric information will be obtained on approximately 400 subjects in the Johns Hopkins School of Medicine, in Baltimore, Md. Since no qualified anthropologist trained in anthropometric techniques is available in the Baltimore area, arrangements have been made to obtain the services of Dr. J. Lawrence Angel of the Anatomy Department of the Jefferson Medical College in Philadelphia. Dr. Angel, an experienced anthropologist, will come to Baltimore twice a week to make the necessary morphological measurements, observations, and somatotype pictures. Cooperation of the Johns Hopkins University School of Medicine has already been established.

6. <u>Budget Plan:</u>	Salaries (Dr. Angel \$35 per diem)	\$2,275.
	Expendable Supplies	387.
	Permanent Equipment	650.
	Overhead (15%)	1,279.
	Other - transportation scheduling-student remuneration, recorder travel, misc.	5,215.

Total \$ 9,806.

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7. Anticipated Duration of Work: One year.

8. Facilities and Staff Available:

Facilities of the Johns Hopkins University School of Medicine  
Dr. J. Lawrence Angel, Anatomy Department of the Jefferson Medical  
College, Philadelphia

9. Additional Requirements:

Principal investigator and co-investigator will receive no salary.

After collection of the morphological data, an additional research grant will be required for the statistical analysis and correlative work-up of the material involving investigators.

10. Additional Information (Including relation of work to other projects and other sources of supply):

For relation of this work to other projects see publications:

- (a) "Masculinity and Smoking" by C. C. Seltzer, Science, (in press).
- (b) "The Precursors of Essential Hypertension and Coronary Artery Disease" by C. M. Thomas and others. Volume 1, 1948-1959. Collected Papers. The Johns Hopkins School of Medicine.

/s/ Carl C. Seltzer, Director of Project

1003541123

C O P Y

TIRC Grant #214 R 1

PEABODY MUSEUM

of

ARCHAEOLOGY AND ETHNOLOGY

Harvard University

Cambridge 38, Massachusetts

November 24, 1959

Dr. Robert C. Hockett  
Tobacco Industry Research Committee  
150 East Forty Second Street  
New York 17, N.Y.

Dear Dr. Hockett:

Following your suggestion I am submitting information necessary for the continuation of the studies now in process, involving (1) the completion of the original project started last April, and (2) the analysis of the questionnaire data of the Harvard graduates of the Class of 1946 as related to their morphological data.

With regard to the original project, namely the study of body build and smoking habits of the Grant Study series of Harvard students, considerable progress has been made since its inception in April 1959. One aspect has already been completed, dealing with the element of masculinity and smoking, and the manuscript is now in press to appear shortly in Science. Somatotype and metrical anthropometry analyses are in process, but have been temporarily delayed owing to our mutually agreed decision to send out a smoking habits questionnaire to over 1,000 members of the Harvard Class of 1946, on whom certain morphological data were obtained by me in 1942. We have already received almost 500 replies, and the present rate of response is running slightly over 50%. Time, effort, and expense in sending out the questionnaire is somewhat in excess of expectation, but the results are satisfactory.

From the present project budget we will have used about \$600 for the questionnaire, about \$200 for traveling expenses in connection with the project and the Thomas-Johns Hopkins survey, and additional sum will be expended for reprints.

In accordance with our recent conversations, then, I am making formal request for an extension of the present project until March 31, 1961, and a grant of \$6900 to cover the budget for this period. This sum includes overhead for Harvard University. With these additional funds and extension of time, the following work will be done:

(1) completion of the original project, the study of body build and smoking habits of the Grant Study Harvard series including somatotype and metrical characteristics.

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(2) a study of the body build and smoking habits of the Harvard Class of 1946 from the present questionnaire and the anthropological data collected in 1942.

I trust this is sufficient information for your committee meeting early in December.

Sincerely yours,

/s/ Carl C. Seltzer, Ph.D.

1003541125

TOBACCO INDUSTRY RESEARCH COMMITTEE

#214R1

Committee:  
Wilson, Chm.  
Little  
Cattell  
(Tagiuri)

150 East Forty Second Street

New York 17, N.Y.

(Activated: 2/15/58  
Renewed: 2/15/59)

Application For Renewal of Research Grant

Date: January 30, 1960

1. Name of Investigator: Carl C. Seltzer, Ph.D.
2. Title: Research Fellow in Anthropology, Peabody Museum
3. Institution  
& Address: Harvard University  
Cambridge 38, Mass.
4. Project or Subject: Study of Body Form with Smoking Habits of  
Harvard Alumni.
5. Detailed Plan of Procedure:
  - A. Completion of original project, the study of body build and smoking habits of the Grant Study Harvard series including somatotype and metrical characteristics.  
  
Study of morphological masculinity and smoking already completed and published. See "Masculinity and Smoking" Science, December 18, 1959, Vol. 130, No. 3390, Pages 1706-1707.
  - B. A study of the body build and smoking habits of the Harvard Class of 1946 from questionnaire data and the anthropological data collected in 1942.  
  
The request for smoking habits data from the Harvard Class of 1946 was most successful, even more so than anticipated. Almost 900 questionnaires were returned.

6. Budget Plan:

Salaries (1/5 time)	\$3,000.
Expendable Supplies	200.
Permanent Equipment	100.
Overhead	900.
Other - statistical services	
clerical services	2,700.
travel, etc.	
Total	\$6,900.

7. Anticipated Duration of Work: To be completed by Feb. 15, 1961.

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8. Facilities and Staff Available: Facilities of Harvard University,  
the Peabody Museum and Statistical Laboratories of Harvard University.
9. Additional Requirements: -----
10. Additional Information (Including relation of work to other projects and  
other sources of supply): -----

/s/ Carl C. Seltzer  
Director of Project

/s/ F. O. Brew, Director  
Peabody Museum

1003541127

TRADOC

February 15, 1960

MEMORANDUM

TO: The Scientific Advisory Board

FROM: Robert C. Hockett

SUBJECT: (1) Publications Resulting from TIRC Projects.  
(2) Other Papers Relevant to TIRC Interests.

We enclose reprints of two papers resulting from TIRC grants-in-aid and four other papers relevant to the interests of the Board:

Papers from Projects:

1. #134B - Nakanishi, Y.H., Mizutani, M. and Pomerat, C.M. Smoke Condensates on Lung Cells in Tissue Culture with Special Reference to Chromosomal Changes. Texas Reports on Biology and Medicine, 17, 542 (1959).
2. #214 - Seltzer, C. C. Masculinity and Smoking. Science, 130, 1706 (1959).

Other Papers:

1. Eastcott, D. F. Other Airborne Factors in Cancer. Abstract of the paper read by Dr. Eastcott at the Symposium on The Air We Breathe at the University of California School of Medicine on Jan. 18, 1960.
2. Kotin, P. Experimental Approach to Lung Cancer. Abstract of the paper read by Dr. Kotin at the same Symposium.
3. Guerin, M. Pulmonary Tumors and Buccal Cancer in Rats Subjected to the Inhalation of Cigarette Smoke. Bull. Fr. Assn. Canc. 46, 295 (1959).
4. Ide, G. A Comparison of the Histopathology of the Bronchial and Tracheal Epithelium of Smokers and Non-Smokers. Gann 49, Supp. 257 (1958).

R.C.H.

RCH:mr  
Encls.

1003541128

See  
Article C44

Reprinted from SCIENCE, December 18, 1959, Vol. 130, No. 3390, pages 1706-1707

Research Fellow in Anthropology

## Masculinity and Smoking

**Abstract.** Study of the relative strength of the masculine component in a series of males reveals a significant association with their differential smoking habits. Weakness of the masculine component is significantly more frequent in smokers than in nonsmokers and most frequent in the heavier smokers.

In order to obtain a fuller understanding of the apparent relationship of heavy smoking to lung cancer and coronary disease, it is pertinent to inquire into the nature of the individuals who practice the smoking habit—their personality, physiology, and biogenetic characteristics. The basic data of the Study of Adult Development (Grant study) of the Harvard University Health Service affords an unusual opportunity for the exploration of some of these factors, in so far as they provide longitudinal smoking information on a group of Harvard alumni over a period of more than 15 years. Portions of this material have already been reported in connection with the psychology of smoking (1) and with a variety of personality, physiological, medical, and social data (2). This report deals with one aspect of the somatic biogenetic material—namely, the masculine component of these men as related to their smoking habits.

The basic data on which this analysis is based are derived from a longitudinal study of 252 Harvard College sophomores first seen between 1938 and 1942, who were selected for their lack of visible abnormality. The details of the project, including the methods, the pro-

cedures, and the nature of the material collected, have been described elsewhere (3). When first seen the subjects were examined for an extensive range of medical, physiological, anthropological, and sociological information. Since then these men have been followed through annual questionnaires, retesting, and visits in order to obtain a variety of factual material, including data on their smoking habits.

A complete description of the collection of the data on smoking has already been presented by Heath (2). The smoking habits of the subjects were recorded during the initial medical examinations made between 1938 and 1942, and the number of cigarettes, pipes, and cigars smoked per day was specified. Subsequently, similar information was obtained from the participants through the medium of annual questionnaires over a period of more than 15 years. From these data it has been possible to construct a threefold classification of nonsmokers (24.3 percent), moderate smokers (38.0 percent), and heavier smokers (37.7 percent), based on the long-term observation of the smoking habits of the subjects.

In the course of the physical anthropological examination of the subjects when they were still college sophomores, between 1938 and 1942, each individual was rated with respect to a body-build complex known as the masculine component (4). The term *masculine component* refers to the element of masculinity in the individual as indicated by his external morphological features. The more the pattern of anatomical traits tends toward the extreme masculine form, the stronger is the masculine component; the greater the departure from the extreme masculine type towards the more feminine build, the weaker is the masculine component in the individual. The gradations from the strong masculine component to the very weak masculine component form a continuum. Nevertheless, with the aid of a standardized chart, individuals may be readily characterized as having a strong, moderate, weak, or very weak masculine component. A description of the morphological traits indicative of the weakness of the masculine component

and illustrations of the various categories have been published elsewhere (4, 5). In practice, the rating of men for strength of the masculine component is relatively simple, and the degree of reliability of the ratings is very high. This is the same element in the morphology of the individual which Sheldon has referred to as gynandromorphy (6).

Table 1 presents the distribution of the individuals in our series according to strength of the masculine component and smoking habits. The data show that there is a significant association between the strength of the masculine component and the smoking habits of the subjects ( $P$  is less than .05) (7). More specifically, weakness of the masculine component is significantly more frequent in smokers than in nonsmokers, and significantly more frequent in heavier smokers than in nonsmokers and moderate smokers combined ( $P$  is less than .05). It is interesting to note that the increased frequency of the degree of weakness of the masculine component from the nonsmokers to the heavier smokers is consistent and progressive. Thus, while only 3.3 percent of the nonsmokers have some degree of weakness of the masculine component, the percentage rises to 9.6 in the moderate smokers and 17.2 in the heavier smokers. At the same time, the heavier smokers show the greatest proportion of individuals with weak or very weak masculine components.

Although these findings are highly interesting and most suggestive, it must be clearly recognized that they should be considered as preliminary and tentative in nature, pending confirmation from future studies designed to illuminate this area of concern.

But the data as they stand lend evidence to the nature of the biogenetic characteristics involved in human behavior, and to the role of the physical constitution in the total personality of the individual. The body-build complex, the masculine component, must be rec-

Table 1. Data showing the relationship between the masculine component and smoking habits ( $N = 247$ ).

Non-smokers		Moderate smokers		Heavier smokers	
No.	%	No.	%	No.	%
<i>Strong masculine component</i>					
58	96.7	85	90.4	77	82.8
<i>Moderate masculine component</i>					
2	3.3	7	7.5	8	8.6
<i>Weak masculine component</i>					
		2	2.1	7	7.5
<i>Very weak masculine component</i>					
				1	1.1
<i>Totals</i>					
60	100.0	94	100.0	93	100.0

**Instructions for preparing reports.** Begin the report with an abstract of from 45 to 55 words. The abstract should not repeat phrases employed in the title. It should work with the title to give the reader a summary of the results presented in the report proper.

Type manuscripts double-spaced and submit one ribbon copy and one carbon copy.

Limit the report proper to the equivalent of 1200 words. This space includes that occupied by illustrative material as well as by the references and notes.

Limit illustrative material to one 2-column figure (that is, a figure whose width equals two columns of text) or to one 2-column table or to two 1-column illustrations, which may consist of two figures or two tables or one of each.

For further details see "Suggestions to Contributors" [Science 125, 16 (1957)].



ognized as a feature of the genotype and as being ostensibly unaffected by environmental considerations. The fact that we find individuals with weakness of the masculine component most heavily represented in the smoking group, and especially in the heavier smoking category, suggests that for a specified type of individual smoking may be a reflection of certain personality and behavioral traits which are characteristic of his biological make-up.

In this connection, it is to be noted that in a previous study the individuals with weakness of the masculine component "exhibit a characteristic pattern of traits which form a consistent and harmonious picture" (4). These less masculine persons tend to have an aversion for strenuous exercise and sports, are apt to be low in physical fitness for hard muscular work, and are often poor in muscular coordination. In the sphere of personality structure, they appear to be more sensitive in affect and manifest a greater degree of instability of the autonomic nervous functions. They are apt to be less well integrated and more ideational, creative, and intuitive. They are more frequently shy and asocial and more frequently have traits of self-consciousness and inhibi-

tion. In the formal intellectual functions they tend to rank higher in the verbal functions and possibly lower in the mathematical or number functions. Academically, they most often select the area of arts, letters, and philosophy as a college major, and their choice of career tends to follow these same lines of interest. What is significant here is the fact that this constellation of personality and behavioral traits for the individuals with weakness of the masculine component is for the most part not inconsistent with the findings of Heath (2) in his study of the differences between smokers and nonsmokers.

If further studies confirm the findings of this report, an important line of investigation should be explored which may bear on the question of the association of smoking with lung cancer and coronary heart disease. In view of the fact that smoking is found here to be significantly more frequent in individuals with weakness of the masculine component, then it would be pertinent to determine the differential frequency of lung cancer and coronary disease in males according to the strength of the masculine component in both smokers and nonsmokers. Such data would help establish whether differences exist in

disease incidence between the classes of individuals within this genotypical body-build complex, and whether the element of smoking materially changes this incidence. Thus, it may be possible to secure evidence on the extent to which smokers and nonsmokers differ in their susceptibility to disease because of their biological nature, apart from the element of smoking itself (8).

CARL C. SELTZER  
Peabody Museum, Harvard University,  
Cambridge, Massachusetts

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5. ——— and L. Brouha, *ibid.* **1**, No. 1, 95, (1943); W. L. Woods, L. Brouha, C. C. Seltzer, *Selection of Officer Candidates* (Harvard Univ. Press, Cambridge, Mass., 1943).
6. W. H. Sheldon, *The Varieties of Human Physique* (Harper, New York, 1940).
7. The statistical significance from which *P* values given in this report are derived is based on the chi-square method of computation.
8. This study was supported by the Tobacco Industry Research Committee and based on data of the Study of Adult Development of the Harvard University Health Service.

13 August 1959

1003541130

CROSS REFERENCE SHEET

Name or Subject

Joseph Shanfeld

Regarding

Research Grant # 8R1

SEE

David E. Mann, Jr.

1003541131

TOBACCO INDUSTRY RESEARCH COMMITTEE  
150 EAST FORTY SECOND STREET NEW YORK 17, N. Y.

Application For Research Grant

Date: **June 12, 1957**

1. Name of Investigator: **Theodore R. Sherrod Ph.D., M.D.**
2. Title: **Associate Professor, Department of Pharmacology**
3. Institution  
& Address: **University of Illinois  
College of Medicine  
1853 West Folk Street  
Chicago, Illinois**
4. Project or Subject: **The Action of Cigarette Smoke on the Dynamics and the  
Metabolism of the Myocardium.**

5. Detailed Plan of Procedure (Use reverse side if additional space is needed):

**Please see additional pages.**

1003541132

6. Budget Plan:

Salaries	Part-time student	1300.00
Expendable Supplies		2500.00
Permanent Equipment		2500.00
Overhead	15%	948.00
Other	workman's compensation on above salary	20.00
Total		7268.00

7. Anticipated Duration of Work:

Duration of present project is anticipated to be 12 months. However, additional studies are likely.

8. Facilities and Staff Available:

Please see additional pages.

9. Additional Requirements:

10. Additional Information (Including relation of work to other projects and other sources of supply):

Our present studies include general cardiovascular work, supported in part by the department of pharmacology and a grant from the United States Public Health Service.

1003541133

Signature /s/ T. R. Sherrod, Ph.D., M.D.  
Director of Project

H.A. Hazleton  
Business Officer of the Institution

/s/ Granville A. Bennett

Most evidence suggests that excessive cigarette smoke inhalation results in marked cardiovascular disturbances in the experimental animal, as well as in the normal person and patient with cardiovascular disease. The cardiovascular effects usually consist of an increase in blood pressure (Graybiel et al., 1938, Russek et al., 1955) and a change in heart rate (Heistand et al., 1940), although in many instances electrocardiographic alterations (Bryant et al., 1947, Graybiel et al., 1938), ballistocardiographic deterioration (Davis et al., 1953, 1955, Henderson, 1953), and occasional cases of tobacco induced angina pectoris have been reported (Boyle et al., 1947, Ralli et al., 1928, Wilson et al., 1939). The mechanism by which cigarette smoke inhalation causes these marked cardiovascular alterations has been studied by numerous investigators. In general, it is agreed that these changes are indicative of myocardial ischemia as a result of either coronary artery spasm or an increase in the work load placed on the heart beyond the capacity of the coronary arteries to supply energy. In a study of angina pectoris due to cigarette smoking Cornwall (1934), Wilson et al. (1930), and Ralli et al. (1938) suggested coronary artery spasm as the cause of the anginal attacks. Allbutt (1915) and Pickering et al. (1945) proposed that the myocardial effects occur as a result of the increased work load placed on the heart. In a ballistocardiographic study Henderson (1953) and Davis et al. (1953) attributed the changes in the ballistocardiographic pattern to coronary vasoconstriction as result of the cigarette smoke. Furthermore, Davis et al. (1956) suggested that the coronary vasoconstriction was due to the release of posterior pituitary hormone as a result of stimulation of the supra optic nuclei by the absorbed nicotine. Wilson and Johnson (1939) and Bellet et al. (1949) suggested that the electrocardiographic changes they observed were due to an increase in cardiac work. Boyle et al. (1947) and Ahn and Gohle (1949) reported that both the increase in cardiac work and the coronary vasoconstriction were responsible for the myocardial effects of cigarette smoke.

In our preliminary experiments a significant increase in cardiac work was not a consistent finding. In several experiments marked electrocardiographic alterations occurred in the absence of a significant change in cardiac work. In addition, no correlation was noted between the severity of the electrocardiographic disturbances and the degree of change in cardiac work. Henderson (1953) and Levy et al. (1947) reported that increases in blood pressure did not occur in all cases in which the cardiac effects were observed, indicating that an increase in cardiac work was not the consistent cause of the observed distress. The lack of correlation between the electrocardiographic and the ballistocardiographic effects of cigarette smoke inhalation, and the increases in cardiac work would tend to limit it as a possible explanation of the changes seen.

Although a decrease in coronary blood flow due to coronary vasoconstriction has been suggested as a possible cause of the myocardial effects of cigarette smoke, there is no evidence to justify this hypothesis. Laubry et al. (1933) measured the coronary blood flow in an isolated rabbit heart and found that nicotine caused an increase in the coronary blood flow. Only in toxic doses was there a diminution of the blood flow in the coronary vasculature. Bulbring et al. (1949) found that administration of cigarette

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smoke to an anesthetized dog resulted in an initial increase in coronary blood flow. This was then followed by a reduction in the coronary blood flow, which was attributed to the release of posterior pituitary hormone containing the antidiuretic and vasopressor factor. Schmitthenner et al. (1956) found that an increased coronary blood flow resulted from the administration of nicotine to the open chest anesthetized dog.

On the basis of this data, it is very doubtful that the effects of cigarette smoke on the heart are due to constriction of the coronary arteries. Although the increase in the work of the heart may contribute to the cardiac effects of smoking, it is doubtful that this is the sole responsible agent, since it is not consistent with the observed responses. The effect of cigarette smoke on the dynamics and the metabolism of the myocardium are not clear and the many clinical manifestations are likewise not well understood. It is for this reason that it seemed worthwhile to study the time course of the dynamic and metabolic changes in the myocardium, including cardiac work, coronary blood flow, and oxygen metabolism in the open chest anesthetized dog.

The following is the experimental protocol and results of our preliminary experiments: Eighteen to 22 kgm. dogs were anesthetized intraperitoneally with pentobarbital sodium (30 mgm./kgm. body weight). Tracheal cannulas were inserted and the animals maintained on room air by means of a positive pressure respirator. A metal sound of small diameter was inserted into the ascending aorta by way of a carotid artery and blood pressure was measured by means of a Statham blood pressure transducer.

A left thorocotomy was performed between the fourth and sixth intercostal space. The pericardium was incised over the left atrium, and the auricular appendage was grasped with a serrefine and retracted from the field. A one centimeter section of the circumflex branch of the left coronary artery, one to three centimeters distal to its bifurcation with the anterior descending branch, was isolated and cannulated with polyethylene tubing. A Shipley rotameter was interposed between this point and a cannulated femoral artery. Heparin sodium was used as the anticoagulant.

Blood pressure, cardiac output, coronary blood flow, and the electrocardiogram were recorded on a Sanborn Multichannel recorder. In addition, several simultaneous leads of the electrocardiogram and arterial blood pressure were recorded on a Grass Multichannel recorder.

The work of the left ventricle was determined making use of the following formula:

$$W = QR + \frac{Mv^2}{2g}$$

W = Work of the left ventricle (kgm. M. per beat)

Q = Mean cardiac output (cc. per beat)

R = Aortic pressure (M. Hg.)

M = Mass of blood ejected (Gms.)

g = 9.8

v/ Velocity of the blood (cc. per sec.)

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Cardiac output was measured by the pulse pressure analysis method of Remington and Hamilton. Blood samples were obtained anaerobically from the aorta, via a catheter, and from the great cardiac vein, which was catheterized by way of the coronary sinus. Blood gas analysis was accomplished by the method of Van Slyke and Niell.

Cigarette smoke was administered by means of a positive pressure pump calibrated to deliver between 1200 and 1500 ml. of smoke in divided quantities over a thirty second period. Measurements were started one minute prior to the administration of cigarette smoke and continued throughout the cardiovascular response.

The results of cigarette smoke administration are indicated in Charts 1 and 2. Chart 1 illustrates the electrocardiographic changes following cigarette smoke administration. Leads 1, 2 and 3 of the electrocardiogram and blood pressure are indicated. The time sequence of each section of the record, in seconds, appears in the lower portion of each section. Chart 2 illustrates the time sequence of the hemodynamic changes due to cigarette smoke. Upon administration of cigarette smoke there occurred an increase in cardiac output, blood pressure, and heart rate. This resulted in a marked increase in cardiac work. After 100 seconds, it was noted that there occurred a marked increase in the oxygen saturation of the coronary venous blood. There was no change in the oxygen saturation of the coronary arterial blood. As a consequence, there occurred a marked decrease in the coronary arterio-venous oxygen difference, hence, reflecting a marked decrease in the cardiac oxygen utilization. Coronary blood flow remained elevated above control levels throughout the experiment. The decrease in the oxygen utilization by the heart in the presence of a markedly increased work load is detrimental to adequate cardiac function is evidenced by the electrocardiographic alterations which occurred in close proximity to this event.

Our preliminary experiments justify further investigation of this problem. The consistency of our findings seem to indicate that the mechanism of the cardiac disturbances as a consequence of cigarette smoke appear to be due to a disturbance in the metabolic processes involved in the oxygen transport mechanism. Further experiments are planned for the further investigation of cigarette smoke, nicotine, and epinephrine.

Our present data indicate that our methods are not sufficient to elucidate a clear definition of the myocardial effects of cigarette smoke. It would be advisable to study the myocardial oxygen tensions so as to clearly define the point at which there is interference with the oxygen utilization. Metabolic studies are also necessary if future experiments continue to indicate a metabolic disturbance. In addition, transmembrane and action potential of the myocardium during the smoke induced period of hyperexcitability would shed light upon its mechanism.

Much of the necessary equipment is obtainable from the Department of Pharmacology. However, our present funds are not sufficient to permit purchase of the necessary equipment and supplies.

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Equipment available:

- 1) Sanborn and Grass multichannel recorders
- 2) Pressure and displacement transducers
- 3) Flow meters
- 4) Surgical instruments
- 5) Beckman DU spectrophotometer with flame attachment
- 6) High gain direct current amplifier
- 7) A polarographic unit

Equipment needed:

- 1) High gain dual beam cathode ray oscilloscope
- 2) A suitable photographic device for recording
- 3) Functional amplifier for computations
- 4) Polarographic electrodes

Expendable Supplies:

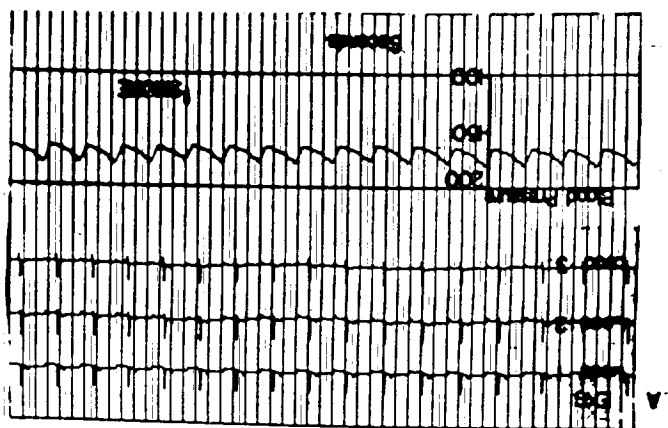
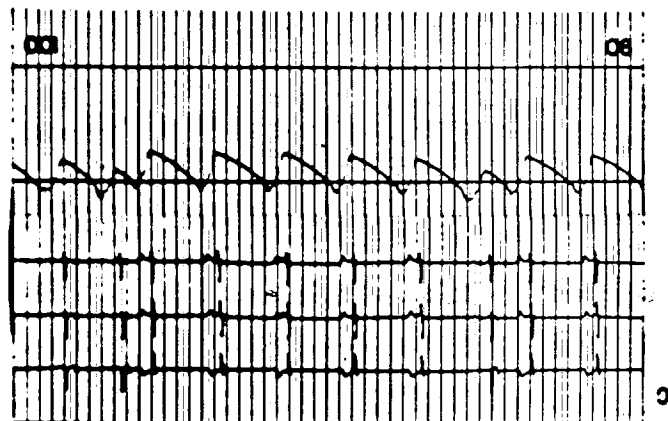
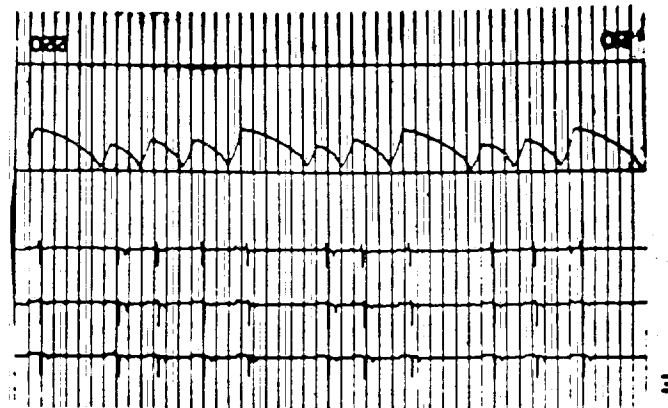
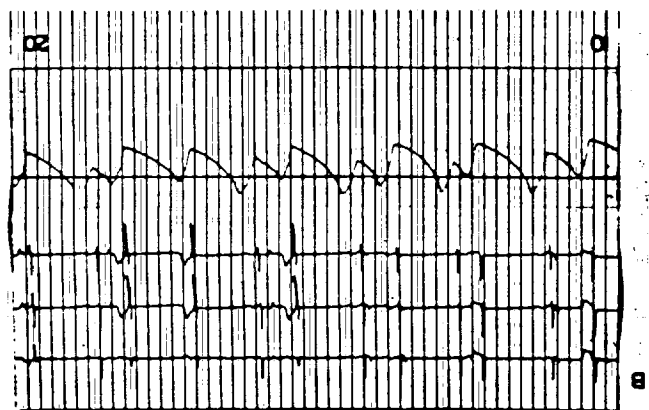
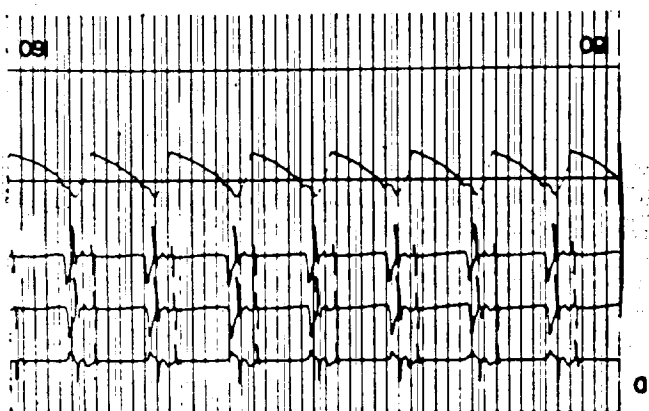
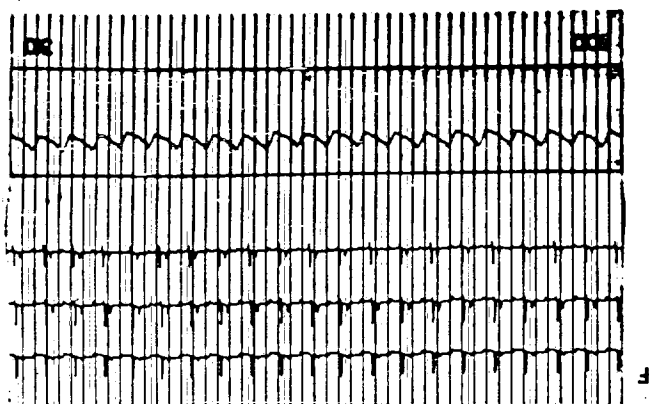
- 1) Animals and care of animals
- 2) Chemicals
- 3) Glassware

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CHART I



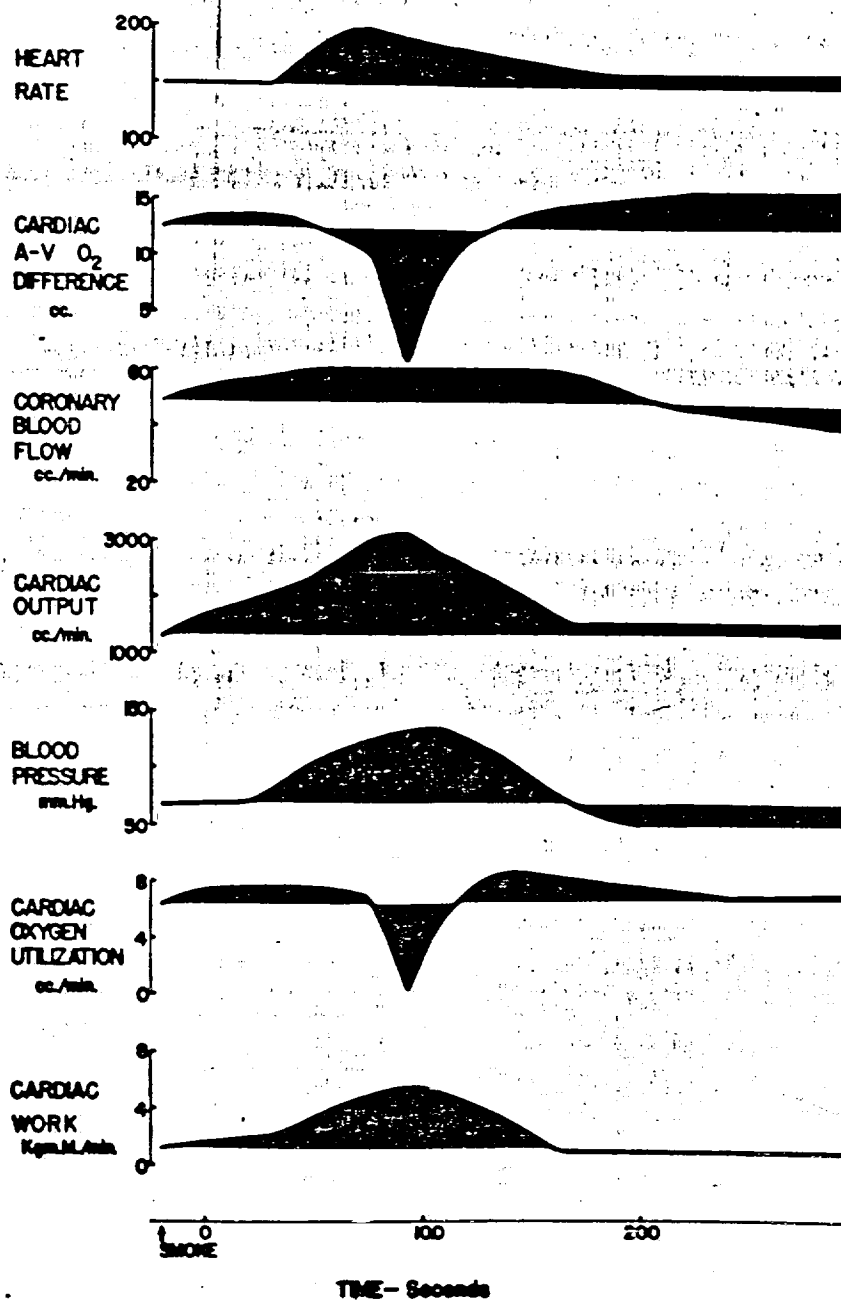


CHART II

1003541139

ACTION OF CIGARETTE SMOKE ON CARDIOVASCULAR HEMODYNAMICS. Gerald A. Kien\*, Norman Lasker\* and Theodore R. Sherrod. Dept. of Pharmacology, Univ. of Illinois College of Medicine, Chicago. Fed. Proc., 16:1,312,1957.

There is considerable controversy concerning the action of cigarette smoke on cardiovascular hemodynamics. This study was designed to determine such actions of cigarette smoke in the open-chest pentobarbitalized dog in which blood pressure, cardiac output, coronary blood flow, cardiac oxygen consumption, cardiac work and the electrocardiographic effects were measured. Immediately following the administration of 1200-1500 cc of cigarette smoke, making use of a standard "king sized" nonfiltered cigarette, the smoke being administered by a specially calibrated smoking device, there was a brief but marked slowing of the heart followed by a sustained pressor response. These effects were attributed to autonomic ganglionic stimulation by the absorbed nicotine from the cigarette smoke. The time course of the hemodynamic responses indicated an early increase in cardiac work which extended over a 3-min period. The coronary blood flow was elevated in relation to the rises in both blood pressure and cardiac output. No independent action of cigarette smoke on the coronary vessels was observed. The coronary arteriovenous oxygen difference decreased markedly at first. This was then followed by a prolonged increase in the coronary arteriovenous oxygen difference. As a consequence, initially the cardiac oxygen utilization was reduced during the period of a greatly elevated cardiac work. This decrease in cardiac oxygen utilization was then followed by a sustained increase. These alterations may be explained on the basis of metabolic changes in the myocardium. That such effects are detrimental to adequate cardiac function is suggested by the extreme electrocardiographic alterations incident to the changes in oxygen utilization during the period of elevated cardiac work.

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1003541141

Application For Research Grant

Date:

Feb. 23, 1955

1. Name of Investigator: Charles E. Sherwood, M.D., Assistant Professor of Radiology  
(Principal Investigator)  
George L. Emerson, M.D., Assistant Professor of Surgery  
Marion S. Emerson, M.D., Instructor of Medicine
2. Title:
3. Institution & Address: The University of Rochester School of Medicine & Dentistry  
260 Crittenden Blvd., Rochester, N. Y.

4. Project or Subject:

Investigation into the natural history of carcinoma of the lung with particular reference to the radiographic appearance of such processes, the earliest manifestation of cancer on chest x-rays and the tabulation of the relationship of smoking habits and occupation with the incidence of lung cancer.

5. Detailed Plan of Procedure (Use reverse side if additional space is needed):

All proven cases of bronchogenic carcinoma seen in this institution since 1926 are to be screened and a ~~xxx~~ careful search made for possible sources of previous chest roentgenograms.

This involves communication with family physician, the patient's family and other hospital and offices having contact with the patient. Any such x-rays are collected and studied for early evidence of cancer or other pertinent disease processes.

*some inaccurately*

A tabulation of the smoking habits is to be made as well as any possible occupational hazard.

From the study of the information tabulated and observed it is hoped that much may be learned about the various early manifestations of cancer of the lung as seen on the chest x-ray. Information relative to smoking habits and occupational exposure for this local area will be available for comparison with other regions and more general statistics.

1003541142

320 EIGHTH AVENUE NEW YORK 17, N.Y.  
TOBACCO INDUSTRY RESEARCH COUNCIL

98

## 6. Budget Plan:

Salaries	\$4,800
Expendable Supplies	200
Permanent Equipment	
Overhead	750
Other	
Total	\$5,750

7. Anticipated Duration of Work: 12 months minimum; in view of previous experience (see item 10) further time seems indicated.

8. Facilities and Staff Available: The investigators are the only personnel necessary. Since our work is mainly investigative with reviewing of films and charts, the facilities of the Department of Radiology are ample.

9. Additional Requirements: None.

10. Additional Information (Including relation of work to other projects and other sources of supply): This project has been in progress for the past 18 months with a local budget to cover postage and mimeographing only. The major basic "footwork" has been and is being done by Dr. Marion Emerson who is not currently in active practice. She is on the part time teaching staff of the University, without stipend. She has had no personal remuneration for her efforts in this endeavor and, indeed, the project is a financial drain on her in the form of household help and transportation. Her professional status is invaluable in initial screening and much time and effort is saved as compared with routine secretarial help. She spends from three to five mornings per week and one evening a week working exclusively on the project. This work entails reviewing case histories, much correspondence with family physicians, patient families, hospitals, radiologists' offices, mobile tuberculosis units, etc., gathering together records and films for review by all of the investigators at a weekly meeting and the tabulation of findings.

No permanent equipment is necessary for the project and, except for postage and printing expenses, we have no other budget necessities.

In the 18 months so far spent we have covered approximately 40% of the cases available at the start of the project. With the passage of time, new cases are added and the recent cases are the more rewarding in terms of long-range x-ray studies and more careful chart recording of personal habits. After the film and case review, effort will be directed toward tabulation of findings. Therefore, it seems probable that more than another year will be required to complete the project.

Signature /s/ Charles E. Sherwood, M.D.  
Director of Project  
Charles E. Sherwood, M.D.

/s/ LaRoy B. Thompson  
Business Officer of the Institution

LaRoy B. Thompson

1003541143

TOBACCO INDUSTRY RESEARCH COMMITTEE  
350 FIFTH AVENUE NEW YORK 1, N. Y.

6. Budget Plan

Salaries  
Expendable Supplies  
Application For Research Grant

Quarters  
Other

Date:

March 12, 1956

#68 RI

1. Name of Investigator:

Charles E. Sherwood, M.D., Assistant Professor of Radiology  
(principal Investigator)

2. Title:

George L. Emerson, M.D., Assistant Professor of Surgery and  
And Assistant Professor of Radiology

3. Institution

Marion S. Emerson, M.D., Instructor of Medicine,

& Address:

University of Rochester School of Medicine and Dentistry,  
260 Crittenden Blv'd.  
Rochester, N. Y.

4. Project or Subject:

5. Additional Information:

Investigation into the natural history of carcinoma of the  
lung with particular reference to the radiographic appearance  
of such processes, the earliest manifestation of cancer on  
chest x-rays and the tabulation of the relationship of  
smoking habits and occupation with the incidence of lung cancer.

5. Detailed Plan of Procedure (Use reverse side if additional space is needed):

6. Additional Information: (Indicate any work on other projects and other sources of help)

The major concern of the investigators is the evaluation of the earliest  
x-ray changes indicative of lung cancer with the aim of earlier suspicion  
and recognition of such lesions. Proven cases of bronchogenic carcinoma are screened and a search made for  
any and all previous chest x-ray. Such a search involves written communication with  
patients, their survivors, family physicians, hospital record departments  
and radiological installations. All radiographs collected are reviewed by the  
investigators. Representative films for possible future publication are being  
photographed prior to their return.

Other statistical information, such as occupational factors and smoking  
habits, is being tabulated.

As indicated by the progress report submitted in February 1956, review  
and analysis of 203 cases had been completed at that time. Collected data on  
250 cases is still before us and we propose to increase this to approximately 300,  
or a total of 500 cases in all. Those already completed are principally  
from the earlier years of the institution. Since most of the remaining cases  
have been discovered either recently or during the past 10 years, information will be  
more complete and the yield of previous radiographs more rewarding than with  
the earlier cases.

Business Office of the Institution

1003541144

6. Budget Plan:

Salaries	
Expendable Supplies	
Application for Repeal of Tax	\$4,800.00
Permanent Equipment	200.00
Overhead	
Other	
Total	750.00
March 12, 1957	\$5,750.00

7. Anticipated Duration of Work:

12 months after completion of current project period. (see item 10)

8. Facilities and Staff Available:

The investigators are the only personnel. Since our work is ~~will~~ mainly investigative with reviewing of films and charts, the facilities of the Department of Radiology are sufficient.

9. Additional Requirements:

Interpretation into the clinical picture of the findings. With particular reference to the clinical picture of the findings, the patient's condition is of primary concern. The patient's condition is of primary concern. The patient's condition is of primary concern.

10. Additional Information (Including relation of work to other projects and other sources of supply):

The major concern of the investigators is the evaluation of the findings. Dr. Marion Baerson continues to be of invaluable aid in her capacity as professional liaison with the patient, family and family physician. Her value in reviewing collected material and culling through the clinical records of patients cannot be overemphasized.

It is proposed to terminate this study on or about July 1, 1957. It would appear reasonable to expect that we will have collected and analyzed approximately 500 cases by the late winter of 1956-57 and the latter months of the project year would be devoted to the evaluation of our findings and the preparation for future publication.

Budget requirements are as before. Amount for expendable supplies is earmarked for postage and the cost of reproduction of any radiographs.

As indicated by the progress report submitted to the Board of Directors and analyzed of 500 cases has been completed at this time. Collected data on 500 cases is still being collected and will be dropped on interest this is approximately 100, to a total of 500 cases in all. These already completed are principally from the earlier years of the investigation. Since work of the past several years has been completed, the field of interest is now being dropped. The field of interest is now being dropped. The field of interest is now being dropped.

Business Officer of the Institution

/s/ LaRoy B. Thompson

1003541145



**CONFIDENTIAL**

TIRC Grant #68

Progress Report #1

Dr. Charles E. Sherwood  
University of Rochester  
School of Medicine and Dentistry

February 10, 1956

"Investigation into the Natural History of Carcinoma of the  
Lung with Particular Reference to the Radiographic Appearance"

This report contains information gathered since the start of the investigation in 1953 and consequently covers more cases than those analyzed in the period of support by the Tobacco Research grant.

A. MATERIAL:

1. A total of 446 cases of cancer of the lung diagnosed during the past 30 years has been gathered and partially analyzed as follows with the year indicating the time of diagnosis.

<u>YEAR</u>	<u>ANALYZED</u>	<u>STILL TO BE ANALYZED</u>	<u>TOTAL</u>
1926	1	2	3
1927	0	2	2
1928	1	4	5
1929	1	2	3
1930	2	1	3
1931	2	3	5
1932	3	3	6
1933	0	6	6
1934	1	5	6
1935	4	5	9
1936	4	3	7
1937	2	7	9
1938	3	5	8
1939	3	8	11
1940	2	8	10
1941	4	5	9
1942	9	7	16
1943	2	5	7
1944	4	0	4
1945	4	4	8
1946	7	3	10
1947	10	5	15
1948	14	11	25
1949	9	7	16
1950	17	9	26
1951	26	15	41
1952	28	25	53
1953	24	8	32
1954	1	38	39
1955	<u>14</u>	<u>23</u>	<u>37</u>
TOTALS	203	243	446

1003541146

2. Not included in this progress report but intended as part of the final summation of the project are:

- a. Tabulation of specific symptoms.
- b. Diagnostic and operative procedures.
- c. Specific pathological cell type.
- d. Tobacco habits.

## B. METHODS:

As of January 1, 1956, 450 letters were written to patients, their families, physicians and to hospitals in an attempt to track down previous x-rays taken prior to the onset of symptoms or prior to diagnosis. Of these, 185 were written in 1955. Other specific information was elicited as indicated. Three hundred answers have been received and the information tabulated.

These letters and a review of records from this and other hospitals lead to the accumulation and study of approximately 600 sets of x-rays. One hundred forty of these were from sources other than our own.

All of the information relative to an individual case has been recorded on a master chart from which statistical information is readily available.

## C. STATISTICS:

Based on 203 currently completed cases. (243 cases are still to be processed plus cases diagnosed in coming months.)

1. Sex: 180 males; 23 females.

2. Age: Average male: 58.2  
Average female: 56.7  
Average total: 58.2

3. Occupation: (known in 162 cases with 167 categories)

<u>Occupation</u>	<u>No. of cases</u>	<u>Per Cent</u>
White Collar	51	30
Housewife	18	11
Miscell. (1 each)	14	8.4
Dust (moulders, etc.)	12	7.2
Machinists	10	6.0
Carpenters	9	5.4
Painters	8	4.8
Tailors	7	4.2
Truck Drivers	7	4.2
Farmers	6	3.6
Janitors	5	3.0
Gardeners	4	2.4
Railroad workers	4	2.4
Garage mechanics	3	1.8
Laborers	3	1.8

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<u>Occupation</u>	<u>No. of cases</u>	<u>Per Cent</u>
Bakers	2	1.2
Plumbers	2	1.2
Teamsters	2	1.2

4. Earliest x-rays found: (whether negative or positive for disease)

Average interval between date of film and diagnosis: 24.3 months  
Extremes: from zero up to 264 months.

5. Earliest x-ray evidence of lung cancer:

a. The average interval between the earliest evidence of cancer and the clinical diagnosis was ----- 4.88 mos.

b. Excluding 58 cases (28%) where no early x-rays were found and in which the x-ray evidence of disease and the diagnosis were simultaneous, the average interval between the earliest x-ray evidence of cancer and the diagnosis ----- 6.9 mos.

c. The average interval between the earliest x-ray evidence of cancer and a definite pathological diagnosis was ---- 5.37 mos.

The longest such interval was ----- 36 mos.

Breakdown of such interval:

	<u>No. Cases</u>	<u>Per Cent</u>
Less than 6 months -----	87	43
6 to 11 months inc.-----	24	12
12 to 23 months incl.-----	22	11
Over 24 months-----	9	4.4
Zero (see C-5-b above)-----	58	28

6. Relationship of symptoms to x-ray change and to diagnosis:

a. X-ray evidence of cancer preceded clinical symptoms in 18 cases (8.8%)

Average interval of such preceding change----- 14 months  
Longest such interval----- 34 months  
Shortest such interval----- 1 month

b. In 16 cases (7.9%) there were no symptoms attributable to cancer of the lung prior to the diagnosis.

c. Duration of symptoms prior to diagnosis (including b above):

Average----- 7 months  
Longest such interval----- 5 years  
Shortest such interval----- 0

Breakdown of interval:

	<u>No. cases</u>	<u>Per Cent</u>
No interval-----	16	7.9
Less than 6 months-----	100	49.4
6 to 11 months-----	46	22.5

	<u>No. cases</u>	<u>Per Cent</u>
12 to 23 months-----	26	12.8
Over 24 months-----	15	7.4

7. The interpretations of radiographs showing the earliest changes compatible with the diagnosis of cancer of the lung have been tabulated in 13 categories as follows:

See Table 1.

D. RESULTS:

No results or conclusions have been, or are intended to be drawn from this preliminary progress report since any such conclusions would be most pertinent after summation of the whole.

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TABLE

## CATEGORIES OF EARLIEST RADIOGRAPHIC CHANGES COMPATIBLE WITH DIAGNOSIS OF CANCER OF LUNG

RADIOGRAPHIC FINDING	TOTAL		FOUND ALONE		WITH 1 OTHER		WITH 2 OTHERS	
	No.	%	No.	%	No.	%	No.	%
Obstructive pneumonitis ** -----	76	37.3	35	17.2	35	17.2	6	3.0
Hilar lymph nodes -----	50	24.6	10	4.9	37	18.2	3	1.5
Parenchymal mass -----	42	20.7						
1 cm. or less in size -----	10	4.9	10	4.9	0		0	
Over 1 cm. in size -----	32	15.8	20	10.1	12	5.9	0	
Hilar mass -----	30	14.8	14	6.9	13	6.4	3	1.5
Atelectasis -----	22	10.8	9	4.4	13	6.4	0	
Pleural effusion -----	19	9.4	0		15	7.4	4	2.0
Parenchymal fibrous infiltrate -----	13	6.4	10	4.9	3	1.5	0	
Mediastinal nodes -----	12	5.9	4	2.0	6	3.0	2	1.1
Parenchymal mass with cavitation ---	6	3.0	5	2.5	1	0.5	0	
Obstructive emphysema -----	5	2.5	1	0.5	4	2.0	0	
Obstructive pneumonitis with breakdown (cavitation) -----	4	2.0	2	1.1	2	1.1	0	
Perihilar infiltration -----	2	1.1	1	0.5	1	0.5	0	
Diffuse parenchymal tumor -----	1	0.5	1	0.5	0		0	

\* a term indicating a combination of atelectasis and infection in a segment of parenchyma.

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*TLC Grant  
#68*

THE NATURAL HISTORY OF  
CARCINOMA OF THE LUNG

GEORGE L. EMERSON, M.D.,  
MARION S. EMERSON, M.D.,  
and

CHARLES E. SHERWOOD, M.D.  
Rochester, N. Y.

From the Departments of Medicine, Radiology,  
and Surgery, The University of Rochester  
School of Medicine and Dentistry

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## THE NATURAL HISTORY OF CARCINOMA OF THE LUNG

*George L. Emerson, M.D., Marion S. Emerson, M.D., and  
Charles E. Sherwood, M.D., Rochester, N. Y.*

IN THE MAJORITY of instances, early diagnosis and ablation of cancer will yield the greatest number of cures. Since the most radical operation practicable is currently used for cancer of the lung, earlier diagnosis should improve the present 3 to 6 per cent cure rate.

Use of clinical diagnostic methods such as bronchoscopy, cytology, scalene biopsy, and thoracotomy have increased in recent years and have helped to make earlier definitive diagnosis. However, a significant change on a roentgenogram of the chest frequently is the first positive finding and often precedes clinical symptoms. The principal objective of this study has been to gain new information about these early changes and, where possible, to observe the alteration in such x-ray shadows with the passage of time. To this end, all roentgenograms obtainable on proved cases of pulmonary cancer have been collected and studied.

### METHOD

Over 400 cases of pulmonary cancer seen in this hospital have been reviewed. This report covers 360 proved cases, the majority having been diagnosed within the past 15 years. Records were abstracted and information was solicited from surviving patients, families, physicians, hospitals, and other institutions where radiographs may have been taken. Any past radiographs available were gathered and reviewed in conjunction with later films made at the time of the diagnosis of the patient's carcinoma. All films were studied in joint session by the authors. Where there was any question of the presence of an abnormal shadow, the opinion of two unbiased senior radiologists was obtained.

### RESULTS

While the radiographic study of these cases has been the prime aim of the project, certain ancillary statistical information is included for general interest with no further elaboration. Tables I through VI list statistics relating to age, sex, occupation, smoking habits, symptoms, diagnostic methods, and cell type.

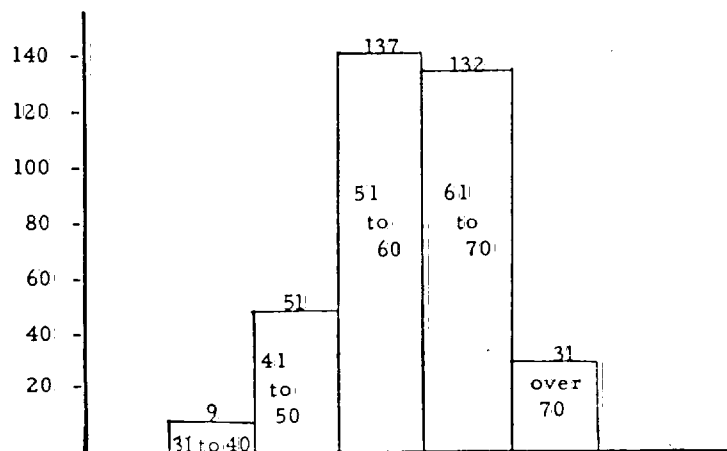
From the Departments of Medicine, Radiology, and Surgery, The University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

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TABLE I. SEX AND AGE DISTRIBUTION IN 360 CASES OF LUNG CANCER

	NUMBER	PER CENT	AVERAGE AGE
Male	313	87	59.4
Female	47	13	56.2
Total	360	100	58.9



Age distribution of patients

TABLE II. OCCUPATIONS IN 360 CASES OF LUNG CANCER\*

OCCUPATION	NUMBER	PER CENT
White collar worker	82	22.8
Housewife	37	10.3
Not known	34	9.4
Hazardous dust exposure	29	8.1
Truckdrivers and mechanics	26	7.2
Laborers	18	5.0
Carpenters	17	4.7
Farmers	15	4.2
Machinists	14	3.9
Painters	12	3.3
Railroad yard and engine workers	9	2.5
Restaurant workers	8	2.2
Tailors	8	2.2
Janitors	6	1.7
Plumbers	5	1.4
Pressers	5	1.4
Garbage collectors	4	1.1
Bartenders	3	0.8
Barbers	3	0.8
Gardeners	3	0.8
Laundry workers	3	0.8
Porters	3	0.8
Stationary engineers	3	0.8
Basket weavers	2	0.6
Firemen	2	0.6
Grocers	2	0.6
Policemen	2	0.6
Printers	2	0.6
Miscellaneous (1 each)	5	0.3

\*Total figure reflects multiple occupations.

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TABLE III. SMOKING HABITS IN 360 CASES OF LUNG CANCER

	WOMEN		MEN		TOTAL	
	NO.	%	NO.	%	NO.	%
No information	18	38	129	41	147	41
Nonsmokers	18	38	7	2	25	7
Group A smokers*	6	13	92	30	98	27
Group B smokers†	5	11	85	27	90	25
Total	47	100	313	100	360	100
	MEN EXPOSED TO DUST‡		MEN EXPOSED TO COMBUSTION FUMES		TOTAL FOR SMALLER GROUP PAST 4 YEARS§	
	NO.	%	NO.	%	NO.	%
No information	9	31	6	23	39	24
Nonsmokers	0	—	0	—	12	7
Group A smokers	7	24	4	15	56	35
Group B smokers	13	45	16	62	55	34
Total	29	100	26	100	162	100

\*Smokers of less than 1 package per day.

†Smokers of 1 package or more per day for 20 plus years.

‡For example, dusts with a potential for producing pneumoconiosis.

§Reflects better recorded information in more recent years.

TABLE IV. OCCURRENCE OF SYMPTOMS IN 360 CASES OF LUNG CANCER

FIRST SYMPTOM			LATER SYMPTOMS		
	NO.	%		NO.	%
1. Cough	194	54	1. Sputum	214	60
2. Chest pain	38	11	2. Chest pain	151	42
3. Weight loss	30	8	3. Hemoptysis	130	36
4. Dyspnea	20	6	4. Dyspnea	109	30
5. No symptoms	19	5	5. Weight loss	89	25
6. Fatigue	17	5	6. Cough	85	23
7. Bone pain	12	3	7. Wheeze	37	10
8. Pleuritic pain	7	2	8. Pleuritic pain	27	8
9. Nerve disorder	7	2	9. Hoarseness	23	6
10. Hemoptysis	5	1	10. Nerve disorder	21	6
11. Hoarseness	4	1			
12. Mass in neck	3	1			
13. Misc. (1 each)	4	1			

TABLE V. DIAGNOSTIC PROCEDURES IN 360 CASES OF CARCINOMA OF THE LUNG

PROCEDURE	NUMBER	PER CENT OF PROCEDURES	PER CENT OF SERIES
<i>Bronchoscopy</i>			
Total examinations	230	100	64
Abnormal appearance	191	83	53
Positive biopsy	99	43	28
Negative biopsy	30	17	11
<i>Scalene Biopsy</i>			
Total examinations	70	100	19
Positive biopsy	46	66	13
Negative biopsy	24	34	7
<i>Papanicolaou Smear</i>			
Total examinations	84	100	23
Positive	63	75	17
Negative	21	25	6
<i>Thoracotomy</i>			
Total operations	150	100	42
Pneumonectomy	60	40	17
Lobectomy	18	12	5
Nonresectable	72	48	20

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TABLE VI. CELL TYPE IN 360 CASES OF CARCINOMA OF THE LUNG

CELL TYPE	MEN	WOMEN	TOTAL	PER CENT
Epidermoid	137	9	146	40
Undifferentiated epidermoid	127	16	143	40
Adenocarcinoma	16	17	33	9
Oat cell	17	0	17	5
Not classified	6	3	9	3
No section (gross appearance; cytology)	10	2	12	3

Table VII is a clinical analysis of 10 patients surviving 5 years or more, an incidence of 2.8 per cent of the total group and 3.1 per cent of the cases seen during the 15-year period in which these survivors were diagnosed.

Table VIII indicates the average interval from the first symptom to clinical diagnosis, to pathologic diagnosis, and to death in varying numbers of patients.

Of major concern in this study is the information tabulated in Tables IX and X. These indicate the average interval elapsing between the earliest radiographic evidence of the individual's cancer and the clinical diagnosis (Table IX) and the pathologic diagnosis (Table X). In each case, this is averaged for four categories: (1) all cases; (2) excluding patients in which the interval between the earliest radiographic evidence and diagnosis was zero; (3) excluding patients in which this interval was less than 3 months; and (4) excluding all cases in which this interval was less than 6 months. Because of the nature of the shadows seen in x-ray studies at the time of diagnosis, it is highly probable that some abnormality would have been present if films had been taken at any point during the 6 months or year prior to diagnosis. Therefore, the more significant is the last group. Rigler and his group have been interested in the duration of pulmonary cancer for some time and have brought out this point.<sup>3, 4</sup>

*Radiographic Findings.*—As previously stated, the major interest in this project has centered around a study of the early radiographic evidences of carcinoma of the lung. In reviewing all earlier films, changes have been sought which could be correlated with the eventual, more gross evidence, of the neoplasm. The types of change seen and their frequency are recorded in Table XI.

1. *Obstructive pneumonitis:* One of the commonest morphologic manifestations of lung cancer, found in 36 per cent of this series, is the encirclement of, or growth into, a bronchus by the neoplasm. This produces a partial obstruction and consequent poor drainage distally. Radiographically, the tumor itself may not be seen while the secondary changes of obstructive pneumonitis are quite common. The term implies a subacute or chronic infiltration with additional evidence of decreased lung volume locally. An illustration of this is given in Figs. 1-4. The inflammatory process is indicated by the heavy linear infiltration at the left base and the partial atelectasis by the elevated diaphragm.

2. *Parenchymal mass:* Twenty-two per cent of the cases had radiographs revealing the direct shadow produced by the more peripherally situated tumor. Illustrations of this are found in Fig. 5, representing a slowly growing lesion, and in Figs. 6-9, representing a rapidly progressive neoplasm. It will be noted

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TABLE VII. CLINICAL ANALYSIS OF 10 CASES OF LUNG CANCER SURVIVING 5 OR MORE YEARS  
AFTER OPERATION

CASE	SEX	INTERVAL FIRST SYMPTOM TO DIAGNOSIS (MO.)	INTERVAL FIRST X RAY CHANGE TO DIAGNOSIS	OPERATION	CELL TYPE	CLINICAL STATUS
B. C.	F	1	1 week	Pneumonectomy, Rt., Oct. 1942	Epidermoid	Moderately decreased pulmonary function
H. C.	M	3	5 months	Pneumonectomy, Rt., Dec. 1950	Epidermoid	Pulmonary status good
J. C.	M	4	2 months	Pneumonectomy, Lt., Dec. 1948	Epidermoid	Died 6 years postop. of unrelated disease
E. E.	M	11	2 months	Pneumonectomy, Rt., Oct. 1951	Epidermoid	Well
C. G.	M	5	4 months	Pneumonectomy, Rt., Sept. 1952	Epidermoid	Well with slightly decreased pulmonary function
L. K.	F	No symptoms	31 months	Lobectomy, Rt., Nov. 1950	Adenocarcinoma	Well; see Fig. 5
J. L.	M	2	1 month	Pneumonectomy, Lt., Jan. 1943	Epidermoid	Moderately decreased pulmonary function
P. L.	M	2	None	Pneumonectomy, Lt., Feb. 1950	Epidermoid	Psychotic; no evidence of recurrence
A. T.	M	16	None	Pneumonectomy, Rt., Apr. 1951	Epidermoid	Died 5½ years postop. of unrelated disease
S. W.	M	5	None	Pneumonectomy, Lt., June 1952	Epidermoid	Died 5 years postop. with probable metastases

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TABLE VIII. AVERAGE INTERVAL BETWEEN FIRST CLINICAL SYMPTOM AND CLINICAL DIAGNOSIS, PATHOLOGIC DIAGNOSIS, AND DEATH IN 359\* CASES OF LUNG CANCER

	NO. OF PATIENTS	AVERAGE INTERVAL (MO.)	EXTREMES OF INTERVAL
Interval between first symptom and clinical diagnosis	359	6.8	0 to 60 mo.
Interval between first symptom and pathologic diagnosis	359	7.8	0 to 60 mo.
Interval between first symptom and death	305	13.8	2 wk. to 104 mo.

\*One case had no recorded clinical history.

TABLE IX. AVERAGE INTERVAL BETWEEN THE EARLIEST RADIOGRAPHIC EVIDENCE OF LUNG CANCER AND THE CLINICAL DIAGNOSIS IN 350\* CASES

	NO. OF PATIENTS	AVERAGE INTERVAL (MO.)
All cases	350	4.0
Excluding 153 cases in which the earliest evidence coincides with the clinical diagnosis	197 (56%)	7.1
Excluding 238 cases in which the earliest evidence was less than 3 months from clinical diagnosis	112 (32%)	11.8
Excluding 276 cases in which the earliest evidence was less than 6 months from clinical diagnosis	74 (21%)	15.9

\*Ten cases of series had no available x-ray studies for review.

TABLE X. AVERAGE INTERVAL BETWEEN THE EARLIEST RADIOGRAPHIC EVIDENCE OF LUNG CANCER AND THE PATHOLOGIC DIAGNOSIS IN 350\* CASES

	NO. OF PATIENTS	AVERAGE INTERVAL (MO.)
All cases	350	5.1
Excluding 81 cases in which the earliest evidence coincides with pathologic diagnosis	269 (77%)	6.1
Excluding 215 cases in which the earliest evidence was less than 3 months from the pathologic diagnosis	135 (39%)	12.2
Excluding 238 cases in which the earliest evidence was less than 6 months from the pathologic diagnosis	92 (26%)	16.1

\*Ten cases of the series had no available radiographs for review.

that, of the ten parenchymal lesions a centimeter or smaller in size, none was associated with any other radiographic change. However, half of those over a centimeter in size were associated with other abnormalities such as hilar enlargement, mediastinal nodes or pleural effusion, an indication of extension of disease. This is a strong argument for early excision of any questionable peripheral lesion.

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3. *Hilar nodule*: In 20 per cent of this series, a subtle increase in size of the hilar shadow was found, frequently best observed by comparison with a previous normal x-ray study. In the majority of instances this change represented the direct evidence of the neoplasm (Figs. 10 and 11).

4. *Hilar mass*: Almost equal in frequency of presenting radiographic shadows was the much more obvious massive enlargement of the hilar shadow, a change making diagnosis much easier and prognosis correspondingly poor.

TABLE XI. TYPES OF EARLY RADIOGRAPHIC ABNORMALITIES FOUND IN 350\* CASES PROVED TO BE LUNG CANCER

RADIOGRAPHIC FINDING	TOTAL		FOUND ALONE		WITH 1 OTHER ABNORMALITY		WITH 2 OTHER ABNORMALITIES	
	NO.	%	NO.	%	NO.	%	NO.	%
Obstructive pneumonitis	129	36.8	60	16.7	56	15.5	13	3.6
Parenchymal mass	77	21.4	45	12.5	24	6.7	8	2.2
Over 1 cm. in size	67	18.6	35	9.7	24	6.7	8	2.2
1 cm. or less in size	10	2.8	10	2.8	0		0	
Hilar nodularity	72	20.0	17	4.7	47	13.1	8	2.2
Hilar mass	69	19.4	35	9.7	25	6.9	9	2.5
Atelectasis	53	14.8	27	7.5	26	7.2	0	
Mediastinal nodes or mass	32	8.9	6	1.7	19	5.3	9	2.5
Pleural effusion	30	8.3	4	1.1	20	5.6	7	1.9
Parenchymal fibrous infiltrate	11	3.1	9	2.5	2	0.6	0	
Perihilar infiltrate	10	2.8	8	2.2	2	0.6	0	
Parenchymal mass: with breakdown	8	2.2	6	1.7	2	0.6	0	
Obstructive emphysema	7	1.9	1	0.3	6	1.7	0	
Lung infection: with abscess	5	1.4	3	0.8	2	0.6	0	
Diffuse parenchymal tumor	2	0.6	1	0.3	1	0.3	0	

\*Ten cases of total series had no available radiographs for review.

5. *Atelectasis*: Atelectasis was found in 15 per cent of the patients. Since all but one of these have died, complete obstruction of a bronchus from a tumor is considered by the authors as a poor prognostic sign. Figs. 12 and 13 illustrate a patient presenting with left lower lobe atelectasis which was thought to be an aortic aneurysm.

6. *Parenchymal infiltrative density*: An example of a carcinoma beginning as a vague fibrous infiltration is shown in Figs. 14 and 15. This change is found with the same frequency as the small parenchymal nodule but is more often confused with old infection.

7. *Miscellaneous*: Less frequent presenting radiographic changes such as fluid, emphysema, abscess, etc., are listed in Table XI.

#### DISCUSSION

An average interval of almost 7 months between the first symptom and the clinical diagnosis emphasizes the continued need for improvement in cancer education of the public and the medical profession.

Again, referring to Tables IX and X, an interval between abnormal x-ray shadows and diagnosis of 4 months for all cases, or almost 16 months for a

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Fig. 1.

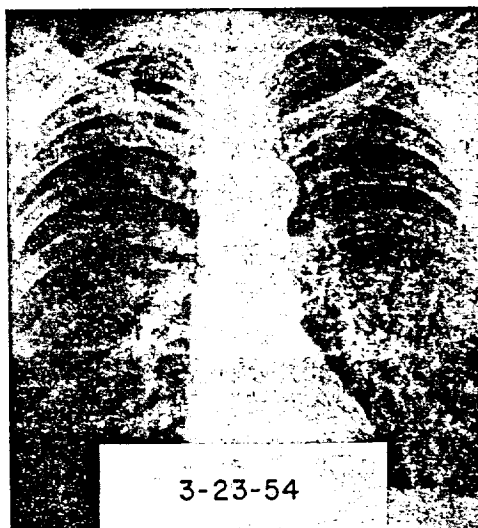


Fig. 2.

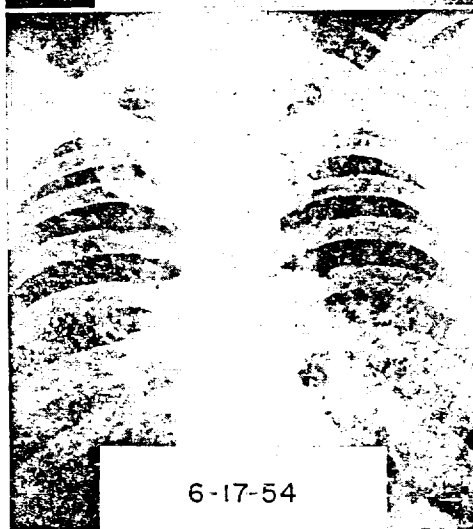
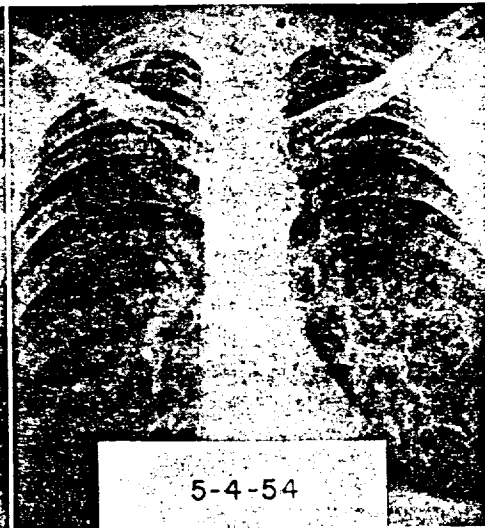


Fig. 3.

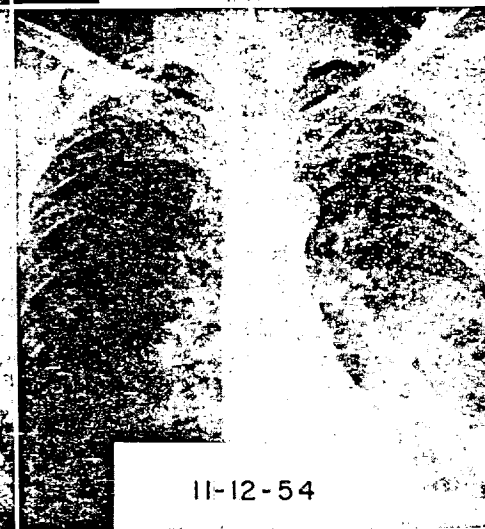


Fig. 4.

Figs. 1-4.—S. D., SMH No. 65317. A 47-year-old former truck driver with cough for the past 20 years which was worse following a bout of pneumonia 3½ years before. He had had frequent bouts of bronchitis. Patient was followed with x-ray studies which were interpreted as chronic pneumonitis for 7½ months during which time he lost 16 pounds in weight. Bronchoscopy and left pneumonectomy on 11/19/54 revealed epidermoid carcinoma. Patient is alive and well as of December, 1957. Progressive obstructive pneumonitis in the left lower lung field is seen in Figs. 1, 2, 3, and 4.

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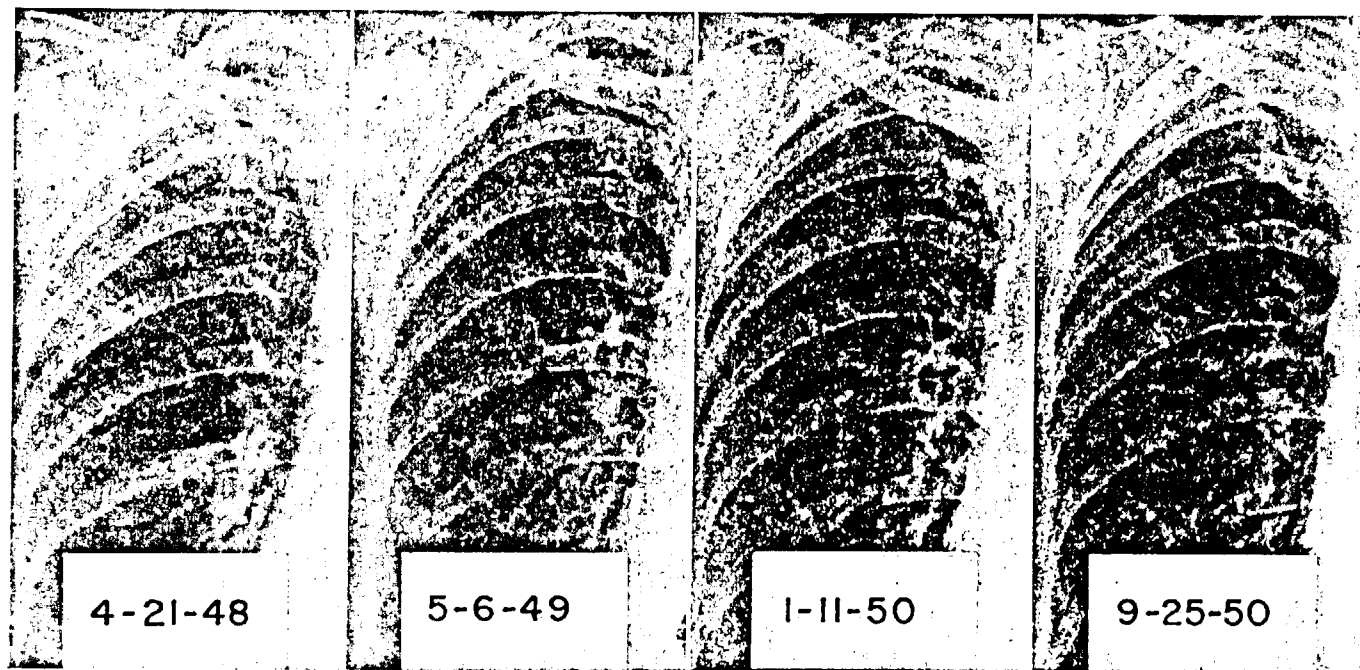


Fig. 5.—L. K., SMH No. 320241. A 53-year-old woman without symptoms. The lesion overlying the third rib anteriorly on film dated 4/21/48 was found on routine annual survey. Growth of the lesion on subsequent films over a period of 2½ years is evident, delay being primarily a medical responsibility. On 11/16/56 a right upper lobectomy revealed an adenocarcinoma. Patient is alive and well as of December, 1957.

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Fig. 6.

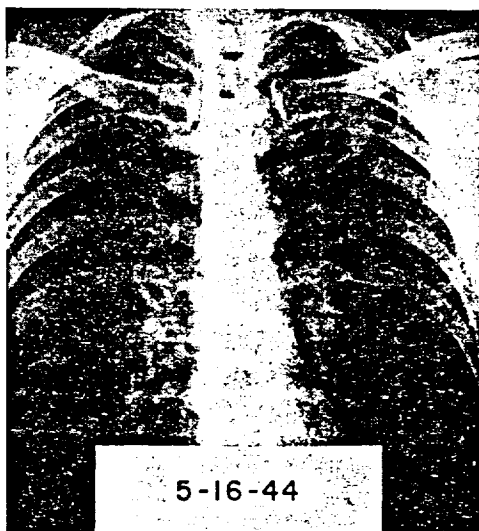


Fig. 7.

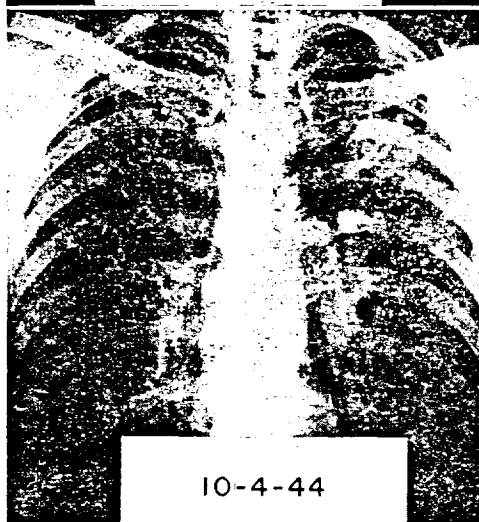
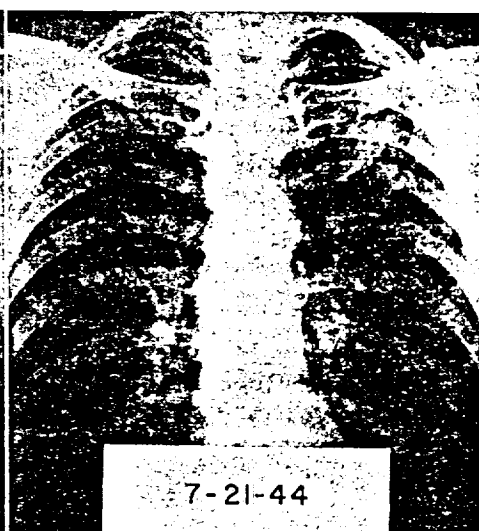


Fig. 8.

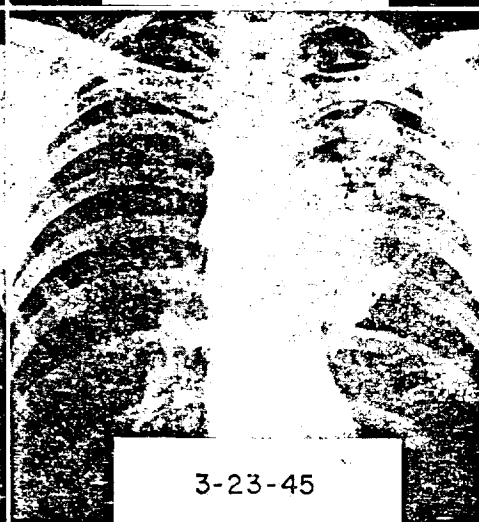


Fig. 9.

Figs. 6-9.—A. H. SMH No. 230290. A 53-year-old male worker in a tuberculosis sanitarium with negative yearly chest roentgenograms from 1937. His first symptom of hoarseness did not occur until 3 months after the film illustrated in Fig. 6. This was followed as tuberculosis without bacteriologic proof. In contrast to the case in Fig. 5, note the rapid growth of the lesion in the left upper lung field; also note rapid enlargement of the hilar shadow and mediastinal extension in 10 months (Figs. 7, 8, and 9).

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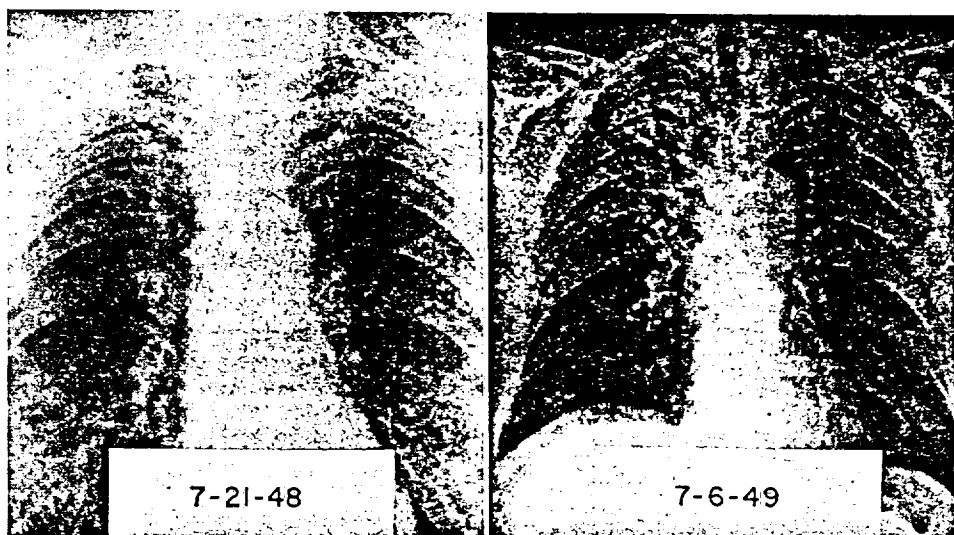


Fig. 10.

Fig. 11.

Figs. 10-11.—R. W., SMH No. 298690. A 66-year-old man admitted July, 1949, with cough, hemoptysis, wheeze, and a 20-pound weight loss during the preceding 2 months. Chest x-ray studies a year before admission (Fig 10) revealed an ill-defined, round density at the upper right hilum. A year later this had enlarged with a considerable component of atelectasis of the right upper lobe as indicated by the elevated hilum and diaphragm (Fig. 11). Biopsy of a cervical lymph node revealed anaplastic carcinoma. Patient died one month following a course of palliative radiation therapy.

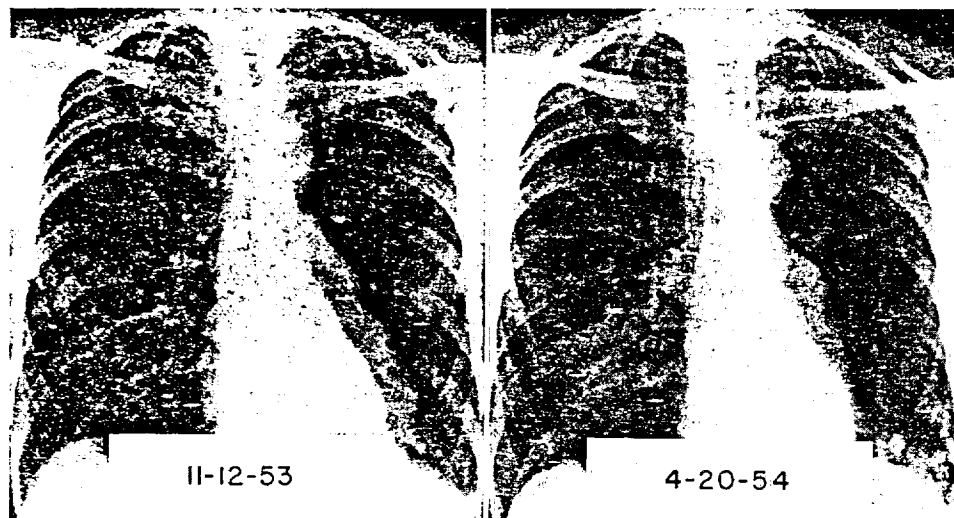


Fig. 12.

Fig. 13.

Figs. 12-13.—M. Mc., SMH No. 181748. A 65-year-old counterman admitted in November, 1954, with increasing cough during the preceding 2 years and a 35-pound weight loss. He had had negative chest films until 11/23/53 (Fig. 12) when an interpretation of thoracic aortic aneurysm was made. The dense shadow along the left heart border is actually an atelectatic left lower lobe with the outline of the heart border seen through it. Five months later (Fig. 13), further collapse is shown with associated emphysema of the left upper lobe. Biopsy at bronchoscopy revealed epidermoid carcinoma and thorotomy proved the lesion to be inoperable. Patient died 9 months later.

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smaller, more significant group, plays a prominent part in the delayed diagnosis of pulmonary cancer. If we are to improve the cure rate, all methods to shorten this interval must be used.

The data relating to the early radiographic findings in cancer of the lung have emphasized that certain changes are highly significant. Hilar, mediastinal and large parenchymal masses, as well as gross atelectasis, produce radiographic shadows strongly suggestive of carcinoma and are of serious prognostic import. Somewhat less obviously produced by a cancer but to be viewed with a high degree of suspicion are obstructive pneumonitis, the small hilar nodule, localized emphysema, and thick-walled irregular abscesses. These carry a slightly less grave outlook, when diagnosed without delay.

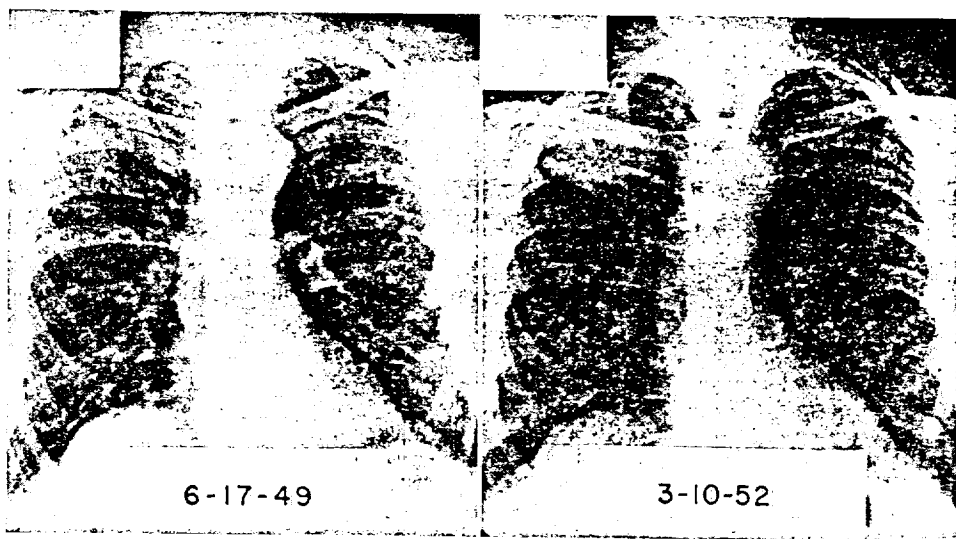


Fig. 14.

Fig. 15.

Figs. 14-15.—J. W., SMH No. 295160. A 58-year-old male reporter admitted in March, 1952, with fatigue, cough, and upper chest pressure. Admission chest film (Fig. 15) revealed a large right apical mass and review of a film taken 2½ years previously (Fig. 14) showed a faint fibrous infiltrate in the first right anterior interspace. At thoracotomy, the lesion was proved to be inoperable and biopsy revealed adenocarcinoma. Patient died 6 months later.

Much has been written recently about the isolated parenchymal nodule which the authors feel is malignant until proved otherwise.<sup>6</sup> As indicated above (Table XI), the smaller the nodule, the less apt it is to have other associated radiographic changes and the better is its prognosis. It is felt that the small persistent infiltrative density should be viewed with almost as much suspicion as the isolated nodule. It will most frequently be confused with a residual fibrous change from inflammatory disease and the distinction, on pure radiographic grounds, is often impossible with a single film. As in the study of changes in the hilar region, the review of current films with any other previous films is of great aid in evaluating the parenchymal nodule and infiltrative density. Even if there are no clues to the radiographic differentiation, the persistent parenchymal infiltrate warrants close follow-up and thorough clinical evaluation. The increase in number of resections now being done for these lesions should yield a better 5-year survival.

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A definite factor in late diagnosis of carcinoma of the lung is the abnormal film which is read as normal. This will happen with even the most experienced eye and will not be completely eliminated. However, all efforts can be constructively directed toward keeping this factor to a minimum. In the case of mass survey examinations, it is frequently not trained radiologists or chest specialists who interpret the films but other physicians, equally loath to miss a diagnosis but without the specialists' experience. In such cases, and even in the established x-ray department, the ideal would include double reading of films, particularly the normal films.

A very minimal change in the hilar shadow is frequently overlooked on a single film where it would be more obvious when compared with a previous normal film. Consequently, when a patient is being studied for possible chest disease, every effort should be made to gather all available past roentgenograms for combined study by the clinician and radiologist. In this regard, the destruction of chest films should be discouraged.

As indicated by Table IX, some 44 per cent of the patients in this series had no film taken prior to the time of diagnosis, or none was available. Yet, the changes present on admission films were such that many patients would have had positive radiographs if films had been taken during the preceding year. An increase in the frequency of films should, therefore, provide earlier case findings. In order to increase the number of chest films taken, the general increase in mass surveys in people over 40 years of age must be supported and patients must be encouraged to obtain periodic x-ray studies of the chest along with physical examinations. Where practical, the individual should be encouraged to have routine x-ray studies done at the same radiologic unit for better comparison follow-up.

Confronted with a suspicious shadow on a chest film and a consequent suspicious lesion in the thorax, it is mandatory that there be continuity of observation and patient care. Increasing numbers of patients suspected of a cancer of the lung today are given the advantage of modern methods of diagnosis. Where the working diagnosis of pulmonary cancer is made more promptly on first recognizing abnormal x-ray shadows, these diagnostic methods will be employed earlier in the course of the disease. It should be emphasized that a high index of suspicion will necessarily make more diagnoses than there are cases and one cannot presume that all suspicious lesions will turn out to be cancer. However, unless one presumes that they might, the toll of bronchogenic carcinoma will not be significantly reduced.

#### SUMMARY

This paper is a study of 360 cases of proved bronchogenic carcinoma with particular reference to the radiographic appearance of such lesions on any past radiographs before definitive diagnosis. Statistical information has been accumulated for general interest.

The average interval of time between early radiographic change and diagnosis for all patients was 4 months. In a selected group (considered more

1003541164

significant) in which radiographs were available more than 6 months before diagnosis, this interval reached the alarming figure of almost 16 months.

The types of early radiographic changes are described. Methods of reducing the time interval between radiographic change and diagnosis and, thus, improving the current low cure rate, are discussed.

The authors wish to express their appreciation to Dr. Roger Terry, Department of Pathology, for his contribution in reviewing the pathologic sections, and to Mr. William Cornwell, of the Eastman Kodak Company, for his help in reproducing some of the radiographs.

#### REFERENCES

1. Hueper, W. C.: A Quest into the Environmental Causes of Cancer of the Lung, Public Health Monograph No. 36, 1955.
2. Overholt, R., Bougas, J. A., and Woods, F. M.: Surgical Treatment of Lung Cancer Found on X-Ray Survey, New England J. Med. 252: 429-432, 1955.
3. Rigler, L. G., O'Loughlin, B. J., and Tucker, R. C.: The Duration of Carcinoma of the Lung, Dis. Chest 23: 50-71, 1953.
4. Rigler, L. G.: Personal communication.
5. Overholt, R., and Bougas, J. A.: Fifty-One Cases of Lung Cancer With Five Year Survival, J. A. M. A. 161: 961-963, 1956.
6. Davis, E. W., Peabody, J. W., and Katz, S.: The Solitary Pulmonary Nodule: A Ten-Year Study Based on 215 Cases, J. THORACIC SURG. 32: 728-771, 1956.

1003541165

TOBACCO INDUSTRY RESEARCH COMMITTEE  
350 FIFTH AVENUE NEW YORK 1, N. Y.

4 Budget Plan

Salaries (and overhead)  
Expendable Supplies  
Application For Research Grant

Overhead  
Other

(travel and so on) Date: September 7, 1954

1. Name of Investigator: Principal Investigator Maurice H. Shulman, M.D.

2. Titles and Staff: Associate Investigators

Brenton R. Lutz, PhD. Professor of Biology  
Chairman of the Department

George P. Fulton, Ph.D. Professor of Biology

3. Institution

& Address:

Department of Biology, Graduate School  
Boston University - 755 Commonwealth Avenue  
Boston 15, Massachusetts

4. Project or Subject:

Direct observations on blood vessels during exposure to the  
constituents of cigarette and pipe smoke.

5. Detailed Plan of Procedure (Use reverse side if additional space is needed).

We propose to use the membranous (mucous cheek pouch of the hamster for the direct observation of blood vessel activity in response to cigarette smoke and for motion picture recording by a technique which has been developed in this laboratory during the past fifteen years. This preparation has given significant results in investigations on thrombus formation, intravascular coagulation, in vivo effects of anticoagulants, vascular effects of corticosteroids, irradiation studies, the mechanism of petechial formation, and the effect of the ingredients of certain skin preparations. This method allows documentation at high magnification (1200 X) with kodachrome motion pictures taken through the microscope by means of transillumination. We have developed methods for taking cheek pouch blood pressures, blood sugars, electrolyte levels, electrocardiograms and blood volumes. We have done these procedures routinely in other projects and correlated them with direct observations.

The following are some of the aspects we plan to investigate:

1. Topical application of the constituents of smoke (paper and tobacco) in the dilutions naturally found.
2. Comparison of the results with the effects on the blood vessels when the same substances are respired.
3. Intravenous administration and also perfusion of these substances in known amounts by a method which by-passes such organs as the liver, lungs and kidneys. Since various substances entering the body parenterally are known to be destroyed or altered by various internal organs, a direct perfusion technique which by-passes the liver, lungs and kidneys may be instructive.
4. Effect of cigarette smoke on the nervous system and heart as shown by encephalography and electrocardiograms.
5. Possible vascular effects of tobacco smoke, such as changes in tendency for thrombus formation, vaso-motor thresholds, vascular fragility, electrolyte levels (notably sodium, potassium, and permeability of cell membranes).

1003541166

Number 5 continued -

6. The preceding studies can be carried out from early life to old age, and the blood pressures followed. The life-span of the hamster is about two years.

7. Because of the short life span, the chronic effects can be determined. In fact, procedures can be carried out and recorded on the same animal from early maturity to old age.

Number 8 continued -

All technical personnel available. Research Associate to be selected from previous doctoral trainees. Special consultants in Biochemistry and Physics are available.

Facilities available:

Laboratories with services and basic equipment. Machine shop and electronic service.

Motion picture camera, projector and dark room facilities

Autotechnicon for tissue work.

Electrocardiographic equipment.

Animal rooms and cages

Equipment for isotope work and dosimetry (permeability studies.)

Electronic stimulator

1003541167

# 6. Budget Plan:

Salaries (and social security, 2%)	\$ 25,468.
Expendable Supplies	2,850.
Permanent Equipment	10,300.
Overhead	3,910.
Other	500.
(travel and communication)	\$ 43,028.00

# 7. Anticipated Duration of Work: 2 years (Second year, \$30,000)

# 8. Facilities and Staff Available:

Principal Investigator	\$ 5,000
2 Associate Investigators	5,000
Research Associate	5,000
Research Assistant	3,800
Photographic Technician	2,000 (part time)
Secretarial Assistant	1,200 (part time)
Animal care	1,000
Consultant fees	2,000
	\$ 25,000
(Please see extra paper)	

# 9. Additional Requirements:

None

# 10. Additional Information (Including relation of work to other projects and other sources of supply):

The proposed research on tobacco constituents is obviously related closely to our current projects (supported by the Public Health Service and the Atomic Energy Commission) such as the production and prevention of thromboembolism, the effects of ionizing radiations on blood vessels, the vascular effects of adrenalectomy and hypersteroidism, and the vascularization of tumors transplanted to the hamster cheek pouch. We have developed various methods for taking chest cavity blood pressures, blood sugar, electrolyte levels, electrocardiograms and blood viscosity. We have done these procedures routinely in other projects and correlated them with direct observations.

The following are some of the aspects we plan to investigate:

1. Topical application of the constituents of smoke (paper and filter) in the hamster cheek pouch.
2. Comparison of the results with the effects on the blood vessels when the same constituents are respired.
3. Intravenous administration and also perfusion of these substances in hamster cheek pouches which bypass the lungs and kidneys. Since various substances entering the body are known to be destroyed or altered by various internal organs, a direct perfusion technique which bypasses the liver, lungs and kidneys may be instructive.
4. Effect of cigarette smoke on the nervous system and heart as shown by encephalography and electrocardiograms.
5. Possible vascular effects of tobacco smoke, such as changes in tendency for thrombus formation, viscosity, blood sugar, electrolyte levels, etc.

Signature: Maurice H. Shulman, M.D.  
Director, Division of Experimental Medicine  
Business Office: Dr. Shulman

1003541168

TOBACCO INDUSTRY RESEARCH COMMITTEE  
350 FIFTH AVENUE, NEW YORK 1, N. Y.Experimental Tobacco  
Society and Foundation  
Application For Research Grant*Probably valuable*

48

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Date: November 29, 1954

## 7. Answer the following questions:

1. Name of Investigator: **Dr. Arthur I. Siegel**

## 2. Title:

**Director**

## 3. Institution:

**Applied Psychological Services**  
5-26 Wilde Ave.  
Drexel Hill, Pa.

## 4. Project or Subject:

The need for the proposed series of investigations into the effects of tobacco on various sensory and motor processes is partially summarized in the recent Tufts College Handbook of Human Engineering Data which states "although tobacco is frequently cited as a possible contributory factor in numerous medical disorders of the sensory processes, the literature is practically devoid of objective studies on the influence of tobacco alone on the sensory mechanisms of normal individuals. Research in this area is greatly needed." (Emphasis ours)

## 5. Detailed Plan of Procedure (Use reverse side if additional space is needed):

A series of psychophysical experiments, using standard psychophysical methodology, into the effects of tobacco on various sensory and motor mechanisms in normal subjects is proposed. The initial experimental work would focus on those areas in which at present little or no data are available. By concentrating our efforts in these virgin areas, a maximum pay-off in applied and basic knowledge is foreseen.

Since nicotine is assumed to depress the action of the nervous structures the following avenues of investigation seem fruitful:

1. An investigation into the effects of nicotine on the stimulus thresholds for pain, cold, warm, and pressure.
2. An investigation into the effects of nicotine on the olfactory stimulus thresholds.
3. An investigation into the effects of nicotine on the ocular motor response.
4. An investigation into the effects of nicotine on auditory thresholds.

(Continued)

Director of the Institution

1003541169



5. An investigation into the effects of nicotine on weight judgment.
6. An investigation into the effects of nicotine on motor response accuracy.

No detailed procedural plan is included with the present application for research grant since (1) the standard methodology proposed is available in all psychological psychophysical reference works, and (2) the methods have been used over the years and seem to be universally accepted. The investigator has taught these methods at both New York University and Queens College and is competent to perform definitive experiments using these methods.

It is not anticipated that all six of the above investigations can be undertaken simultaneously. The budget that follows is based on one year's work, during which two to three of the above experiments can be completed. Prior to the second year the completed research can be reviewed and a decision reached in regard to continuation of the project.

1003541170

## 6. Budget Plan:

TOBACCO INDUSTRY  
350 FIFTH AVENUE  
Salaries  
Expendable Supplies  
Permanent Equipment  
Applied Overhead  
Other

Salaries	\$10,000.00
Expendable Supplies	200.00
Permanent Equipment	480.00
Applied Overhead	1,000.00
Other	500.00
<b>Total</b>	<b>\$12,180.00</b>

Date:

November

## 7. Anticipated Duration of Work:

1. Name of Investigator

Two years

Dr. Arthur L. Steward

## 8. Facilities and Staff Available:

2. Title

Applied Psychological Services has on its permanent professional staff men possessing all the abilities needed to carry the above investigations through from experimental design to final statistical treatment of data and report of results in the professional literature. Appropriate space possessing all the requisites for controlled experimental work will be available after 1 January, 1955.

## 9. Additional Requirements:

1. Name of Institution

None

## 10. Additional Information (Including relation of work to other projects and other sources of supply):

5. Brief Plan of Procedure (Use reverse side if additional space is needed)

The proposed investigator is currently involved with projects involving the physiological and psychological effects of various newly developed pharmaceutical preparations. In addition he has published in the professional theoretical and experimental literature in the areas of sensory and discriminative. Three of these articles are appended in order that a better appraisal of his work can be made.

Kindle nicotine is assumed to depress the activity of the nervous system. The following program of investigation was suggested:

1. An investigation into the effects of nicotine on the sensory threshold for pain, cold, heat, and pressure.
2. An investigation into the effects of nicotine on the sensory threshold for taste.
3. An investigation into the effects of nicotine on the sensory threshold for smell.
4. An investigation into the effects of nicotine on the sensory threshold for touch.

Signature

Director of Project

(Continued)

Business Officer of the Institution

1003541171

TOBACCO INDUSTRY RESEARCH COMMITTEE  
150 EAST FORTY SECOND STREET NEW YORK 17, N. Y.

#187

Application For Research Grant

Date: December 13, 1957

1. Name of Investigator: **Herbert Silvette, B. S., M. S., Ph. D.**
2. Title: **Visiting Professor Pharmacology**
3. Institution  
& Address: **Medical College of Virginia  
Richmond 19, Virginia**
4. Project or Subject: **Immunological Investigation of Tobacco and Tobacco Smoke**

5. Detailed Plan of Procedure (Use reverse side if additional space is needed):

A survey of the literature (H. Silvette, P. S. Larson & H. B. Haag, "Immunological Aspects of Tobacco and Smoking," American Journal of Medical Sciences 234:561-589, 1957, discloses that virtually no fundamental experimental work on animals has been performed in connection with the immunology of tobacco. That tobacco, or its extracts or smoke, may elicit positive skin reactions in man which are true antigen - antibody reactions has been unequivocally demonstrated, which encourages belief in the probability that, under optimal conditions and in susceptible species, antibody responses to tobacco antigens may also be elicited and studied in animals.

The program for such an investigation will be essentially a classic study of the immunology of any presumed antigenic substance. The first step will be to actively sensitize rabbits and guinea pigs to (1) tobacco, both green and cured, of various strains; (2) tobacco extracts prepared in various ways; (3) tobacco smoke; and (4) extracts of tobacco smoke. Standard intravenous injection schedules will be used, and also subcutaneous and intraperitoneal injection of the several materials prepared with Freund's adjuvant. In the case of tobacco smoke, the attempt will be made to actively sensitize guinea pigs through tobacco-smoke exposure, and also to shock passively sensitized guinea pigs by inhalation of tobacco smoke. From time to time, rabbit immune serum

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will be tested for precipitins against one or more antigens, and the animals themselves tested for Arthus and skin reactions. Guinea pigs will be given challenging injections of various antigens. Once a workable technique has been evolved for good immunization, and a potent rabbit immune serum available, cross-immunization and cross-precipitin tests will be carried out using suitable extracts of varieties of tobaccos, smoking forms, and commercial brands of cigarettes. In addition, the immunology of tobacco pollen and tobacco seeds will be investigated.

While there is no experimental evidence that nicotine per se is antigenic, there are a number of observations which indicate that nicotine may influence immune reactions. An attempt will be made to determine whether nicotine may act as a haptene. That this may occur in disease in man, through combination with some body protein, has been suggested as a possibility. Experimentally, active immunization of animals with nicotine deliberately so combined, would be of very considerable theoretical and clinical importance, if successful. A number of techniques to test this possibility have been tentatively formulated. Animals immunized with such nicotine-protein complexes will be challenged with the original nicotine-protein mixture, the protein alone, and nicotine alone.

1003541173

6. Budget Plan: **(First year)**

Salaries	12,125
Social Security	1,650
Expendable Supplies	500
Permanent Equipment	1,475
Overhead	300
(Travel)	
Other	
Total	16,225

7. Anticipated Duration of Work: **Two years**

8. Facilities and Staff Available: **Animal quarter and caging facilities, centrifuge, constant temperature baths, incubators, etc., needed for the type of work proposed. The support of the pharmacology staff as needed.**

9. Additional Requirements: **In the salary item listed in (6) are budgeted \$7500 for the principal investigator, \$4000 for a trained technician and \$600 for a part time animal man.**

10. Additional Information (Including relation of work to other projects and other sources of supply):

The principal investigator has been interested in immunopharmacology for many years, and in 1952-53 was head of the Allergy Research Laboratory, University of Virginia Medical School. Further research in immunopharmacology was carried out during 1953-54 in the Department of Pharmacology, University of Washington Medical School. Publications in the field of immunology include: "Antigen system of acacia," Fed. Proc. 12:1210, 1953 (H. Silvette and O. Swineford, Jr.). "A protein precipitating polysaccharide from acacia," Fed. Proc. 13:1329, 1954 (H. Silvette). "Observations on acacia as an immunizing, sensitizing, anaphylactogenic, and desensitizing antigen," J. Allergy 26:509, 1955 (H. Silvette, O. Swineford, Jr. and L. Tull).

Concerning supplies of tobacco materials for this project, it is hoped that many if not all of the various tobacco fractions that will be studied can be obtained from the Tobacco Industry.

Signature Herbert Silvette  
Director of Project

W. F. Tompkins  
Business Officer of the Institution  
Comptroller

TOBACCO INDUSTRY RESEARCH COMMITTEE  
150 EAST FORTY SECOND STREET NEW YORK 17, N. Y.

#148

(Compare #63 initiated 4/15/55.  
Completed.)

Application For Research Grant

Date: February 5, 1957

1. Name of Investigator: David L. Simon, M.D., Arnold Iglauer, M.D. and John R. Braunstein, M.D.
2. Title: Instructor in Medicine and Fellow in Cardiovascular Research, Department of Internal Medicine, College of Medicine, University of Cincinnati
3. Institution Cincinnati General Hospital, Cardiac Laboratory, B-4  
& Address: Cincinnati 29, Ohio
4. Project or Subject: The Effects of Pipe Smoking and Cigar Smoking on the Cardiovascular System of Man.

This is a continuation of our interest in the field of tobacco and the cardiovascular system. The first paper was entitled "The Immediate Effect of Cigarettes on the Circulation of Healthy and Habitual Male Smokers" which appeared in the American Hrt. J. 48:185, 1954.

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Our most recent work was done under grant #3619 of the Tobacco Industry Research Committee and will be published soon in the Journal of the American Medical Association.\* A complete report of this work has been submitted to Robert C. Hockett, Associate Scientific Director.

\*Will appear in Feb. 2, 1957  
issue of J.A.M.A.

5. Detailed Plan of Procedure:

Male subjects of various ages both normal and with heart disease will be studied on 3 consecutive days in the following manner: they will be placed in the post-absorption state in a constant temperature room at the Kettering Laboratory, University of Cincinnati and after a period of equilibrium in a room of controlled humidity, dew point and temperature will be given either placebo consisting of a pipeful of special tobacco with most of its nicotine removed or their regular pipe tobacco and after an average pipe smoking period the following observations will be made: observations will be made automatically at short intervals of skin temperature, of toes and fingers and forehead. Blood pressures and pulse rates will also be recorded at short intervals. When maximum changes have occurred and the record becomes normal or near normal the patient will be asked to return the next day for the same procedure except with the opposite form of tobacco (that is, if he smokes regular tobacco in his pipe the first day he will be given low nicotine tobacco the 2nd day and vice versa). In addition, the same observations will be made on male subjects with cigars and on 1 day the cigar will be smoked through a silica-gel filter, and on the other day the subject's brand cigar will be smoked without a filter.

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#### 5. Detailed Plan of Procedure (continued)

The third day each subject will be taken to the ballistocardiographic laboratory located in the Cincinnati General Hospital and part of the Cardiac Laboratory of the Department of Internal Medicine, University of Cincinnati. The ballistocardiograms will be recorded at short intervals; a resting period, a smoking period, and a post-smoking period. During a short time the patient will smoke either his regular pipe tobacco in his pipe or will smoke his regular cigar.

When enough patients have been studied to make statistical analysis valid the data, as in our other paper, will be submitted to statistical analysis.

The proposed study in essence will be carried out essentially as our former studies were above except that we will be studying pipe smoking and cigar smoking.

1003541176

## 6. Budget Plan:

Salaries	2,500.00
Expendable Supplies	300.00
Permanent Equipment	
Overhead (5%)	140.00
Other	
Total	2,940.00

7. Anticipated Duration of Work: 6 months to a year

8. Facilities and Staff Available: The facilities of the Cardiac Laboratory, Department of Internal Medicine, University of Cincinnati, and the facilities of the Kettering Laboratory of Applied Physiology, University of Cincinnati. The immediate staff would consist of myself, Dr. Arnold Iglauer, Mrs. Beatrice Doherty of the Kettering Laboratory, Dr. Raymond Suskind of the Kettering Laboratory, Dr. John Braunstein of the Cardiac Laboratory, Mr. Robert Schwemberger, technician of the Ballistocardiographic Laboratory, and Miss Norma Grimm and Mrs. Betty Boyd, secretaries of the Cardiac Laboratory.

9. Additional Requirements: ~~My~~ None

10. Additional Information (Including relation of work to other projects and other sources of supply):

This is a continuation of our studies and our interest in tobacco. We are helped by grants from the National Heart Institute of the United States Public Health Service, and donations from local industries and local individuals.

Signature David L. Simon  
Director of Project

Robert Hoefler  
Business Officer of the Institution



TOBACCO INDUSTRY RESEARCH COMMITTEE  
350 FIFTH AVENUE NEW YORK 1, N. Y.

6. Budget Plan:

Salaries Doctors, Secretaries, etc.	630.00
Expandable Supplies	100.00
Application For Research Grant	
Overhead	
Other	

Date: February 4, 1955

1. Name of Investigator: David L. Simon, M.D., Arnold Iglauer, M.D., and John Braunstein, M.D.

2. Title: Instructor in Medicine and Fellow in Cardiovascular Research, Department of Internal Medicine, College of Medicine, University of Cincinnati, Cincinnati, Ohio  
3. Institution: Cincinnati General Hospital, Cardiac Laboratory, B-4, Miss Helen  
Address: Cincinnati 29, Ohio  
Physician, Secretary and Receptionist: Dr. Raymond Suckin  
and Mrs. Jean Peterson, Receptionist

4. Project or Subject: The effects of chewing tobacco on the cardiovascular system of man.

5. Additional Requirements:

5. Detailed Plan of Procedure (Use reverse side if additional space is needed)

Male subjects of various ages, both normal and with heart disease are being studied on 3 successive days in the following manner: They are placed in the post-absorption state in a constant temperature room at the Kettering Laboratory of Applied Physiology and after a period of equilibrium in a room of controlled humidity, due point, and temperature are given either placebo consisting of special tobacco sent to us by the P. Lorillard Company with most of its nicotine removed or a regular chewing tobacco and after a good chew are asked to spit it out. Observations are made automatically at short intervals of skin temperatures of the toes, fingers, and forehead. Blood pressures and pulse rates are recorded. When the maximum changes have occurred and the record becomes normal or near normal the patient is asked to return the next day for the same procedure except with opposite form of tobacco (that is, if he chews regular chewing tobacco the first day he would be given low nicotine tobacco the next day and vice versa).

The third day the patient is taken to the Ballistocardiographic Laboratory located in the Cincinnati General Hospital and part of the Cardiac Laboratory of the Department of Internal Medicine, University of Cincinnati, and ballistocardiograms are recorded at short intervals during a resting period, a chewing period, and a post-chewing period, during which time the patient chews only regular tobacco.

When enough patients have been studied to make statistical analysis valid, the data, as in our first paper on the smoking of cigarettes, will be submitted to statistical analysis.

Director of Project

In addition leading authorities in the field of peripheral vascular diseases will be and have been circularized (the ones already contacted have shown keen interest) on their experience with the effects of chewing on peripheral vascular diseases such as Burger's Disease and other diseases of the arteries and veins of the extremities.

Business Officer of the Institution

Comptroller

100354178

## 6. Budget Plan:

Salaries	doctors, secretaries, technicians	\$2500.00
Expendable Supplies		300.00
Applicable Permanent Equipment		-----
Overhead		-----
Other		-----
Total		\$2800.00

7. Anticipated Duration of Work: 6 months to a year

8. Facilities and Staff Available: The facilities of the Cardiac Laboratory, Department of Internal Medicine, University of Cincinnati, and the facilities of the Kettering Laboratory of Applied Physiology, University of Cincinnati. The immediate staff would consist of myself, Dr. Arnold Iglauer, Mrs. Beatrice Doherty of the Kettering Laboratory, Dr. Raymond Suskind of the Kettering Laboratory, Dr. John Braunstein of the Cardiac Laboratory, Miss Helen Rothier, secretary and technician of the Ballistocardiographic Laboratory, and Mrs. Betty Boyd and Mrs. Jean Patterson, secretaries of the Cardiac Laboratory.

9. Additional Requirements: The effects of chewing tobacco on the peripheral vascular system of man.

10. Additional Information (Including relation of work to other projects and other sources of supply): None

10. Additional Information (Including relation of work to other projects and other sources of supply): This is a continuation of our studies and our interest in tobacco. We are helped by grants from the National Heart Institute and of the United States Public Health Service and donations from local industries and local individuals. The patient is given a cigarette containing a thermocouple which is sent to us by the P. Bullard Company with most of its plastic removed or a cigarette containing tobacco and after a good time we asked to spit it out. Measurements are made periodically at short intervals of skin temperatures of the toes, fingers, and forehead. Blood pressure and pulse rate are recorded. When the abnormal changes have occurred and the heart becomes normal or near normal the patient is asked to return the next day for the same procedure except with opposite form of tobacco (that is, if he chews regular chewing tobacco the first day he would be given low nicotine tobacco the next day and vice versa).

The patient is taken to the Ballistocardiographic Laboratory located in the Cincinnati General Hospital and part of the Cardiac Laboratory of the Department of Internal Medicine, University of Cincinnati, and ballistocardiograms are recorded at short intervals during a baseline period, a chewing period, and a post-chewing period, during which time the subject chews only regular tobacco.

Even though patients have been studied to make statistical analysis valid, the above, in our first paper on the smoking of cigarettes, is being submitted to statistical analysis.

In addition leading authorities in the field of peripheral vascular diseases will be approached (the ones already contacted have shown keen interest) on their experience with the effects of chewing on peripheral vascular diseases such as Burger's Disease and other diseases of the arteries and veins of the lower extremities.

Signature: /s/ David I. Simon  
Director of Project

/s/ Robert W. Hoefler  
Business Officer of the Institution

Comptroller

TIRC Grant #148 (cf. #63)

David L. Simon, M.D.  
Arnold Iglauer, M.D.  
Cincinnati General Hospital

*Project*  
**CONFIDENTIAL**

*A.E.O.:*  
*Can we learn anything about how important nicotine is to the smoker from these results. Maybe Borden can make an estimate of the reliability of his vs. low nicotine data reported here.*

November, 1959

The Effects of Pipe Smoking and Cigar Smoking on the Cardiovascular System of Man

Much attention has been devoted to the effects of smoking cigarettes on the circulation but relatively little information is available on the circulatory action of pipe and cigar smoking. Such information seems important because of large amounts of tobacco consumed in the United States in these forms. In 1955, it was estimated there were 8.8 million pipe smokers, and the current annual consumption of pipe tobacco nearly 75 million pounds. (1,2) In 1955, there were 10.6 million cigar smokers who consumed close to 6.75 billion cigars. (1,2) In a recent report of mortality experience of nearly 200,000 United States Government Life Insurance policy holders, although an increased death rate was noted in heavy cigarette smokers, the rate in regular cigar and/or pipe smokers was not appreciably higher than that of non-smokers. There was no indication that regular cigar and pipe smokers experience a higher death rate from cardiovascular disease than non-smokers, and the death rate from lung cancer in cigar and pipe smokers is only slightly higher than in non-smokers. (3) Similar findings were reported in an earlier study sponsored by the American Cancer Society. (4) Therefore, it seemed worthwhile to gather data on the circulatory changes caused by smoking regular and low nicotine cigars and pipe tobacco and to compare the results with our own previous studies on cigarette smoking and chewing tobacco, (5,6) as well as with other studies.

Method of Study

The subjects in this study were 25 habitual male smokers. Fifteen subjects smoked cigars and ten smoked pipes; their ages ranged between 21 and 69 years. Studies were carried out in the basal post-absorptive state on each subject on three separate days. On one day low nicotine tobacco was smoked in a constant temperature room, and on another day regular tobacco was smoked. On a third day the effect of high nicotine tobacco on the ballistocardiogram was determined; the order of studies was variable. Skin temperatures were measured in the constant temperature room at 78 Degrees F. (25.6 C.) and 40% relative humidity from copper-constantan thermocouples attached to the forehead and the volar surfaces of the fingers and toes, employing a Brown constant recording potentiometer. Changes of 0.5 degrees C. were considered significant. Blood pressures and pulses were obtained by the usual clinical method. The Ballistocardiograms were recorded with our high frequency table type research instrument. (7) The subjects were supine and unclothed and rested for at least 45 minutes prior to smoking. The period of smoking was 20 minutes. The commercial cigars were Garcia y Vega Roosevelts, reported to contain 1.82% nicotine (moisture

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free basis). (8) The low-nicotine cigars were Sano, containing, according to the manufacturer 0.88% nicotine. The pipe tobacco used was Half and Half, containing 2.15% nicotine (moisture free basis). (8) Sano pipe tobacco containing 0.72% nicotine was used for the low nicotine studies.

The low nicotine cigars weighed 6.6 grams, the regular cigars weighed 6.5. A pipeful of tobacco weighs approximately 2.0 grams. For comparison, a standard cigarette contains 1 gm of tobacco and approximately 20 mg (2%) nicotine. (9)

## Results

### Ballistocardiogram

Cigars: Studies were made with commercial cigars in 12 subjects. In 9 subjects, ballistocardiograms were normal before smoking. Three of these remained normal throughout the smoking period and after smoking. Three showed increased respiratory variation. Three, however, showed greater changes from normal, either in the form of the early M or the late down-stroke patterns.

The records of the other 3 subjects were abnormal before smoking. In two of these, the abnormality increased during and after smoking, and in one it improved. The maximum changes occurred within 10 to 20 minutes of the start of smoking and persisted for 20 to 30 minutes or longer.

In summary, 3 of the 12 subjects showed no change in the ballistocardiogram after smoking, 3 showed only borderline changes, and in 5 abnormality appeared or increased after smoking. One patient's ballistocardiogram improved with smoking.

Pipes: Ten subjects were studied while smoking commercial tobacco. The ballistocardiograms of 8 were normal before smoking. Three of these remained normal throughout; three showed only minor changes, consisting either of minor respiratory variation or changes in the amplitude of the complexes. Two showed rather marked abnormality after smoking, with development of a definite early M pattern.

In 2 subjects who smoked pipes, the records were abnormal before smoking. In one of these, the record showed only minimal changes during smoking but in the other the abnormalities became much more marked. The changes occurred in from 10 to 20 minutes and persisted for 20 or 30 minutes or longer.

In summary, the ballistocardiograms of 3 pipe smokers remained unchanged during smoking, 4 showed only borderline changes and in 3 abnormalities appeared or increased during the test.

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### Pulse Rate

Pulses were measured in 15 subjects who smoked both high and low nicotine content cigars. In all of the subjects smoking commercial cigars, the pulse rate increased; the range of increase was 1 to 12 beats per minute, with an average increase of 6.4 beats per minute. During the smoking of the low nicotine cigars, the pulse rate increased in 14 subjects, the range being from 1 to 15 beats more per minute. The average increase in these subjects was 5.9 beats per minute. In one subject, the pulse rate decreased slightly while smoking a low nicotine cigar.

Pipes: Ten subjects smoked regular and low nicotine tobacco in pipes. During the smoking of the commercial tobacco, 9 subjects showed an increase in pulse rate, ranging from 3 to 20 beats per minute, with an average increase of 7.6 per minute. In one subject the rate decreased 4 beats per minute. While smoking low nicotine tobacco, the pulse rate increased in 7 subjects with a range of two to twelve beats per minute. Average increase was 7.0 beats per minute. In two subjects, the heart rate decreased 4 and 10 beats per minute while smoking low nicotine tobacco and in one the rate remained unchanged. Maximal changes in the pulse rate occurred from 2 to 30 minutes after the start of smoking.

### Blood Pressures

Cigars: Fifteen subjects smoked regular and low nicotine cigars. All subjects showed an increase in both systolic and diastolic blood pressure with smoking regular cigars. The systolic blood pressure increased an average of 7.3 mm. Hg., range 2 to 12 mm., and the diastolic blood pressure increased an average of 8.9 mm. Hg., range 2 to 16 mm.

During the smoking of low nicotine cigars, systolic blood pressure increased in 11 subjects, the average increase being 6.5 mm. Hg., range 1-15 mm. In 3 there was no change in systolic blood pressure, and in one the systolic blood pressure decreased. Diastolic blood pressure increased in all subjects with an average increase of 6.3 mm. Hg., range 2 to 16 mm.

Pipes: Blood pressures were measured in 10 subjects while smoking commercial and low nicotine tobacco in pipes. During the smoking of high nicotine tobacco, the systolic and diastolic blood pressure increased in all subjects. The average increase was 9 mm. systolic and 8 mm. diastolic with a range of 1 to 18 mm. Hg. increase. During the smoking of low nicotine tobacco in pipes, systolic blood pressure increased in 5 subjects, decreased in 2 and remained unchanged in 3. Diastolic blood pressure increased in 7 of these patients and decreased in 3.

### Skin Temperatures

Cigars: Skin temperatures were measured in the fingers and toes of 15 subjects who smoked commercial cigars. The average initial temperature of the fingers was 32.3 degrees Centigrade. Skin temperatures fell in 12 of

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the subjects, remained unchanged in one and increased slightly in 2. These changes are presented graphically in figure 1. Eleven subjects were studied with low nicotine cigars. The initial fingertip temperatures averaged 32.5 degrees Centigrade. In 9 subjects, the skin temperatures decreased, and in 2 subjects, changes were equivocal. (Figure 1.) In both groups of observations, the toe temperatures did not seem to show any consistent pattern of change.

Pipes: Skin temperature observations were made on 10 subjects while smoking commercial tobacco in pipes. The average initial temperature of the fingers was 33.2 degrees Centigrade. Finger temperatures decreased in 9 of the subjects and in one subject, changes were equivocal. (Figure 2.) Nine subjects were studied with low nicotine tobacco. The average initial finger temperature was 33.9 degrees Centigrade. Finger temperatures decreased in 8 of the subjects, and in one subject, changes were equivocal. No consistent pattern of changes was noted in the feet. Maximal changes in skin temperatures occurred from nine to fifty minutes after the start of smoking.

#### Discussion

In our own studies (5) and those of Henderson (10) of the ballistocardiograms during smoking cigarettes, no changes were observed in healthy young males. Other observers have found changes in a small proportion of healthy and abnormal subjects while smoking cigarettes (11, 12). In contrast, we found deterioration of the ballistocardiogram in twenty-three of twenty-four subjects who chewed tobacco (6). Abnormalities of the ballistocardiogram occurred in five of twelve subjects after smoking cigars and in three out of 10 subjects after smoking pipes. Thus, the incidence of ballistocardiograph change with pipe and cigar smoking was higher than that observed with cigarette smoking, but lower than that observed with chewing tobacco.

Increases in pulse rate while smoking cigars were rather moderate in comparison with those reported by Roth et al (13) after smoking two cigarettes and in comparison with our own findings with chewing tobacco. Little difference was found between the commercial and low nicotine cigars. The increases in pulse rate while smoking either regular or low nicotine tobacco in pipes were likewise rather small. No significant difference was noted between pipes and cigars.

Although systolic and diastolic blood pressure increased in all subjects who smoked commercial cigars the changes were less than those generally noted with smoking cigarettes and tobacco chewing. The blood pressure changes were less marked and less consistent with the smoking of low nicotine cigars. The pattern of blood pressure change found while smoking pipe tobacco was very similar, with less consistent changes again noted during the smoking of low nicotine tobacco.

Skin temperature changes after smoking regular cigars and pipe tobacco were about equal, with slightly greater decrease during cigar smoking. If

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our findings are compared with the reported studies of Roth (14) Englehardt and Stuttgen (15), and Mayer and Maddock (16) of skin temperature changes produced by cigarette smoking, the patterns are similar, with slightly greater decreases in cigarette smokers. When the changes following cigar and pipe smoking in the present study are compared with our previous study of chewing tobacco, it is found that maximum changes were slightly greater following tobacco chewing.

Previous studies are not in agreement as to the percentage of nicotine absorbed by smokers who do not inhale. The most frequently cited work, that of Baumberger (17) in 1923 indicates a high percentage of nicotine absorption, even without inhaling (66-77%). A more recent study by Greenburg and others (17) indicates that only a small percentage of nicotine content of smoke is retained in the body without inhaling. They found only 4 to 45% of the nicotine was retained in smokers who did not inhale, whereas 96-98% was absorbed in subjects who inhaled. In our study, none of the subjects inhaled while smoking cigars or pipes. Nevertheless, the pattern of changes observed was similar to that reported in patients inhaling cigarette smoke.

Thus, the present study indicates that failure to inhale does not prevent appearance of the usual circulatory effects of nicotine. Very few previous studies of pipe and cigar smoking were found. Maddock and Collier (19) in a few subjects found following pipe smoking that pulse, blood pressure and skin temperature changes were similar to cigarette smokers. According to Scott (20) nicotine levels in the blood were lower after cigar or pipe smoking than after cigarette smoking.

It should be pointed out that the amount of nicotine available for absorption varies with such factors as moisture content (17, 18) combustion temperature (17), length of filtration, loss through and expectoration, as well as the obvious factors of nicotine content of tobacco smoked and rate of smoking.

#### Summary and Conclusions

1. Ballistocardiograms, blood pressure, pulse rates and skin temperatures were studied in volunteer cigar and pipe smokers during and after smoking.
2. The usual effects of tobacco on the circulation were found (i.e. increase in pulse and blood pressure, drop in skin temperature), but were not as marked as usually found following cigarette smoking or chewing tobacco.
3. The incidence of changes in the Ballistocardiograms in this study was less than that found following chewing tobacco, but greater than that found following cigarette smoking.
4. The circulatory effects of cigars and pipe smoking were approximately the same.
5. Circulatory changes were somewhat less following low nicotine cigars and pipes when compared to standard cigars and pipes.

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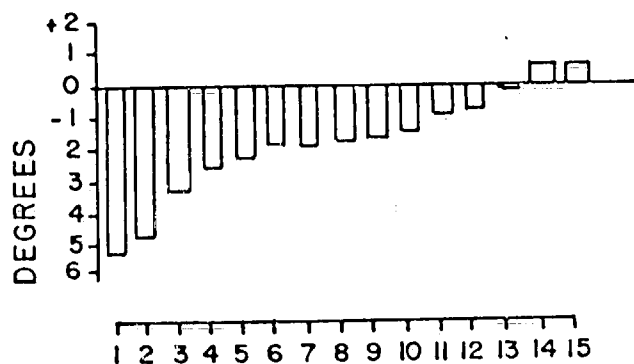
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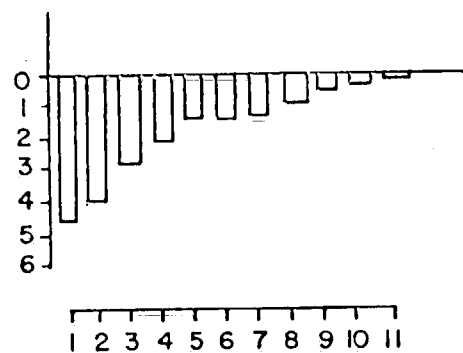
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FINGERS HIGH CIGARS

FIG. 1



FINGERS LOW CIGARS

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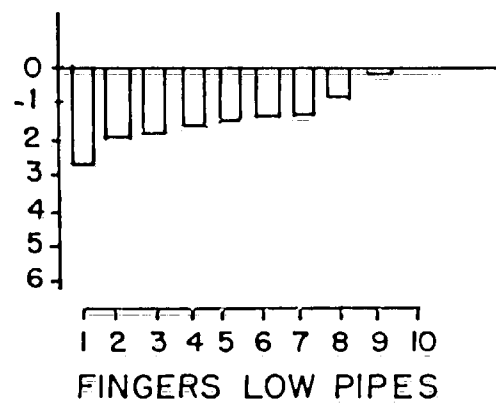
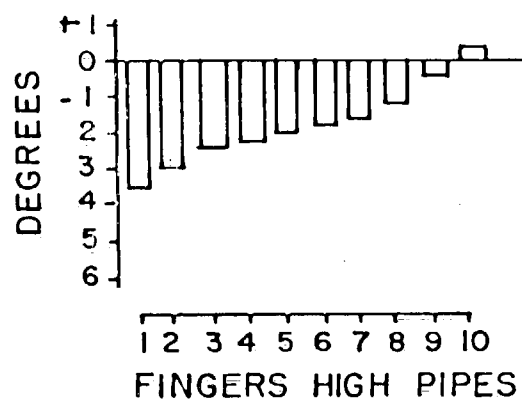


FIG.2

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CONFIDENTIAL

TIRC Grant #148 (Cf. #63)

Progress Report No. 4

David L. Simon, M.D.  
Arnold Iglauder, M.D.  
Cincinnati General Hospital

February, 1960

The Effects of Pipe Smoking and Cigar Smoking on the  
Cardiovascular System of Man

Much attention has been devoted to the effect of smoking cigarettes on the circulation but relatively little information is available on the circulatory action of pipe and cigar smoking. Such information seems important because of the large amounts of tobacco consumed in the United States in these forms. In 1955, it was estimated there were 8.8 million pipe smokers; the current annual consumption of pipe tobacco is nearly 75 million pounds. In 1955, there were 10.6 million cigar smokers; in 1959 almost 6.75 billion cigars were smoked. It is interesting that in two recent reports of mortality experience of nearly 400,000 men, although an increased death rate from carcinoma of the lung and heart disease was noted in heavy cigarette smokers, the death rate in regular cigar and/or pipe smokers was not appreciably higher than that of non-smokers. Therefore, it seemed worthwhile to gather data on the circulatory changes caused by smoking regular commercial and low nicotine cigars and pipe tobacco.

Method of Study

The subjects in this study were 25 habitual male smokers; their ages ranged between 21 and 69 years. Eight subjects were studied while sham smoking unlit pipes or cigars under identical conditions. Studies were carried out in the basal post-absorptive state. Skin temperature changes were measured in each subject on different days while smoking regular commercial or low nicotine tobacco. On a third day the effect on the ballistocardiogram of smoking regular commercial tobacco was determined; the order of these studies was variable. Skin temperatures were measured in a constant temperature room at 78° F. (25.6 C.) and 40% relative humidity from copper-constantan thermocouples attached to the forehead and the volar surfaces of the fingers and toes. Blood pressure and pulse rates were obtained by the usual clinical method. The ballistocardiograms were recorded with a high frequency table type research instrument. The subjects were supine and unclothed and rested for at least 45 minutes prior to smoking. The period of smoking was 20 minutes. The commercial cigars were Carcia y Vega Roosevelts, containing 1.82% nicotine. The low nicotine cigars were Sano, containing 0.88% nicotine. The pipe tobacco used was Half-and-Half, containing 2.15% nicotine. Sano pipe tobacco containing 0.72% nicotine was used for the low nicotine studies.

The cigars weighed approximately 6.5 gms. A pipeful of tobacco weighs approximately 2.0 gms. For comparison, a standard cigarette contains 1 gm. of tobacco and approximately 2% nicotine.

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## Results

### Ballistocardiogram:

Cigars: Studies were made while smoking regular commercial cigars in 12 subjects. In 9 of the 12 subjects, ballistocardiograms were normal before smoking. Three of these remained normal throughout the smoking period and after smoking. Three showed increased respiratory variation. Three, however, showed greater changes from normal, either in the form of early M or late downstroke patterns.

SLIDE I - The records of the other 3 subjects were abnormal before smoking. In two of these, the abnormality increased during and after smoking, and in one it lessened. The maximum changes occurred within 10 to 20 minutes of the start of smoking and persisted for 20 to 30 minutes or longer.

Pipes: Ten subjects were studied while smoking regular commercial tobacco. The ballistocardiograms of 8 were normal before smoking. Three of these remained normal throughout; three showed only minor changes, consisting either of minor respiratory variation or changes in the amplitude of the complexes. Two showed rather marked abnormality after smoking, with development of a definite early M pattern.

SLIDE II - In 2 subjects who smoked pipes, the records were abnormal before smoking. In one of these, the record showed only minimal changes during smoking but in the other the abnormalities became much more marked. The changes occurred in from 10 to 20 minutes and persisted for 20 or 30 minutes or longer.

SLIDE III - Pulse Rate:

SLIDE IV - Blood Pressure:

SLIDES V, VI, VIII -

### Skin Temperatures:

The average initial skin temperature of the fingers was 32.3° C. Skin temperature in forehead did not change. Changes in toe temperatures were variable and inconsistent.

## Discussion

### Ballistocardiogram:

Previous observers have found changes in only a small proportion of healthy young males during cigarette smoking. We found deterioration of the ballistocardiogram in 23 of 24 subjects who chewed tobacco. Abnormalities of the ballistocardiogram occurred or increased in 8 of 12 subjects after smoking cigars and in 6 of 10 subjects after smoking pipes.

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In contrast to studies of cigarette smokers, none of these subjects inhaled. Previous studies are not in agreement as to the percentage of nicotine absorbed by smokers who do not inhale. The most frequently cited work, that of Baumberger in 1923 indicates a high percentage of nicotine absorption, even without inhaling (66-77%). A more recent study by Greenberg and others indicated that without inhaling a smaller percentage of nicotine content of smoke is retained in the body. They found 4 to 45% of the nicotine was retained in smokers who did not inhale, whereas 96-98% was absorbed in subjects who inhaled. This raises the question of the effect of the respiratory activity alone during smoking - on the circulation.

Mulinos and Shulman found that deep breathing could produce transient vaso-constriction in the hands. However, none of our pipe or cigar smokers inhaled or breathed deeply. Many previous observers: Roth, Maddock and Collier, Wright and Moffatt, Weatherby - have shown that sham smoking produced negligible changes in skin temperatures, pulse rates and blood pressures. More recent studies by Freund were confirmatory. Furthermore, Weatherby found quite inconsistent, small and variable changes in actual respiration during the act of smoking. In our own studies of sham smoking, no changes were found even in subjects who showed changes while smoking.

Thus, the present study indicates that failure to inhale does not prevent appearance of the usual circulatory effects of nicotine. Very few previous studies of pipe and cigar smoking were found. Maddock and Collier, in a few subjects, found - following pipe smoking - that pulse, blood pressure and skin temperature changes were similar to cigarette smokers. According to Scott, nicotine levels in the blood were lower after cigar or pipe smoking than after cigarette smoking.

It should be pointed out that the amount of nicotine available for absorption varies with such factors as moisture content, combustion temperature, length of filtration, loss through expectoration, as well as the obvious factors of nicotine content of tobacco smoked and rate of smoking.

#### Summary and Conclusions

The usual effects of tobacco smoking on the circulation were found following smoking of both regular and low nicotine tobacco in cigars and pipes; that is, pulse and blood pressure increased and skin temperature fell in the fingers. Changes were not as marked as usually found following cigarette smoking or chewing tobacco.

The incidence of changes in the ballistocardiograms in this study was less than that found following chewing tobacco, but greater than that found following cigarette smoking.

The circulatory effects of cigar and pipe smoking were approximately the same.

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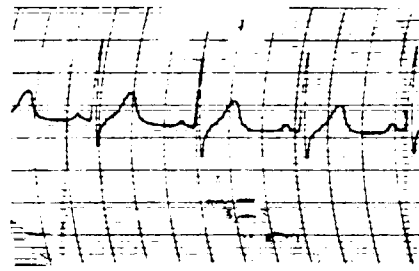
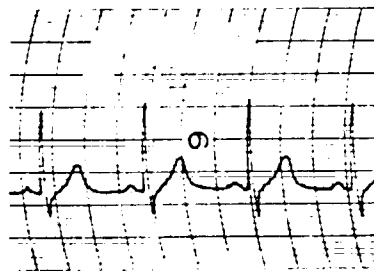
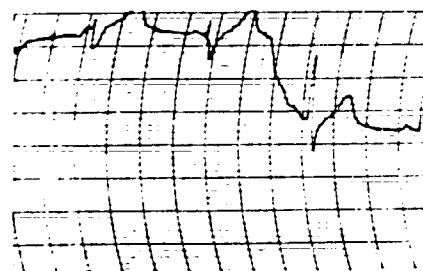
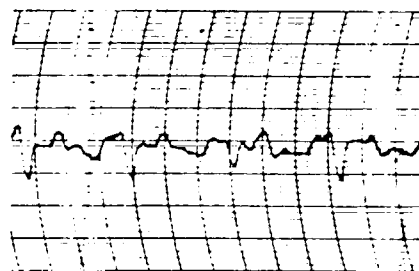
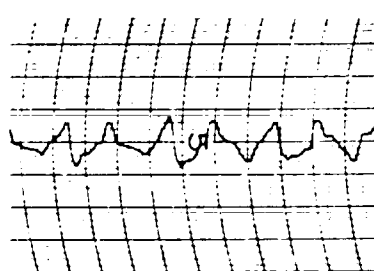
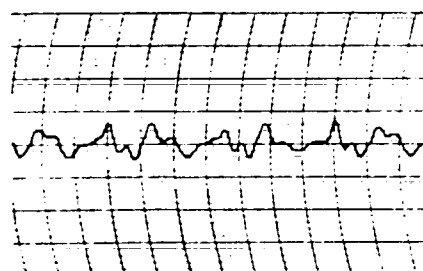
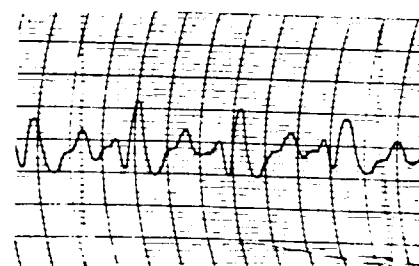
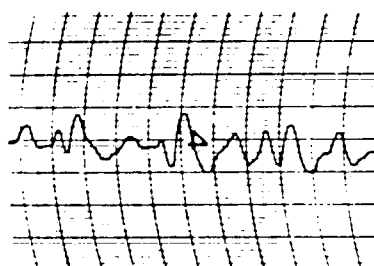
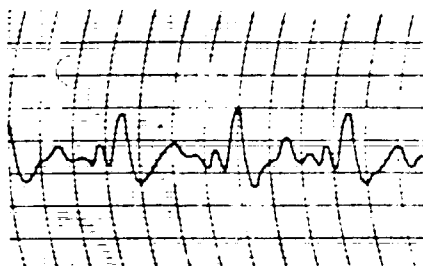
Summary and Conclusions (cont'd)

Circulatory changes were slightly less following low nicotine cigars and pipes when compared to standard cigars and pipes.

In describing the slides - comparisons were made in slides 3, 4 and 7 of cigar and pipe smoking and cigarettes and chewing tobacco.

Slides 5 and 6 were like the others but don't have corrected prints, so enclose them anyway.

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Control

11 Minutes

44 Minutes

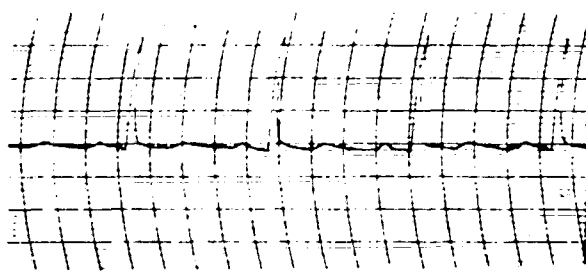
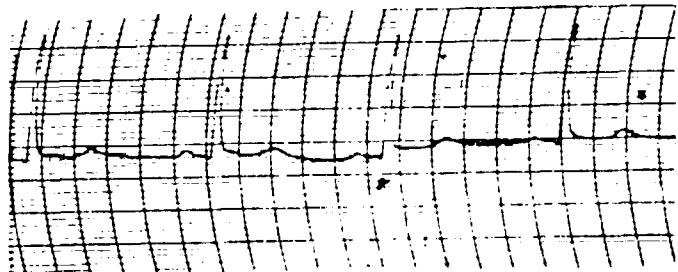
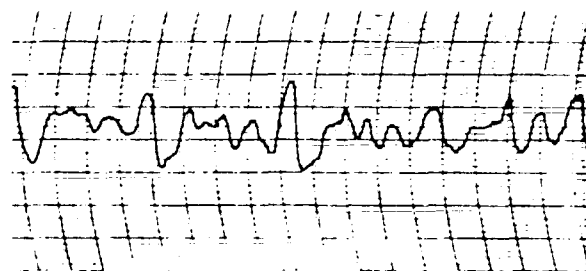
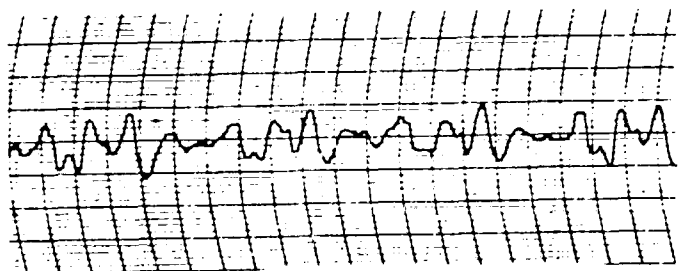
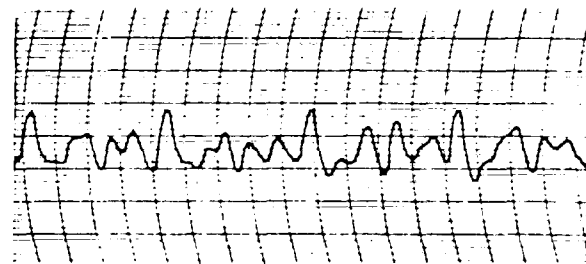
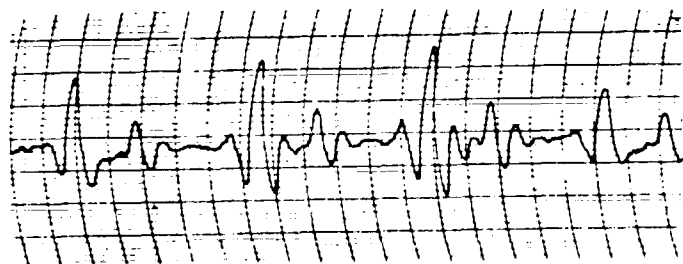
BALLISTOCARDIOGRAMS CIGAR

SMOKING

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slide 2





**Control**

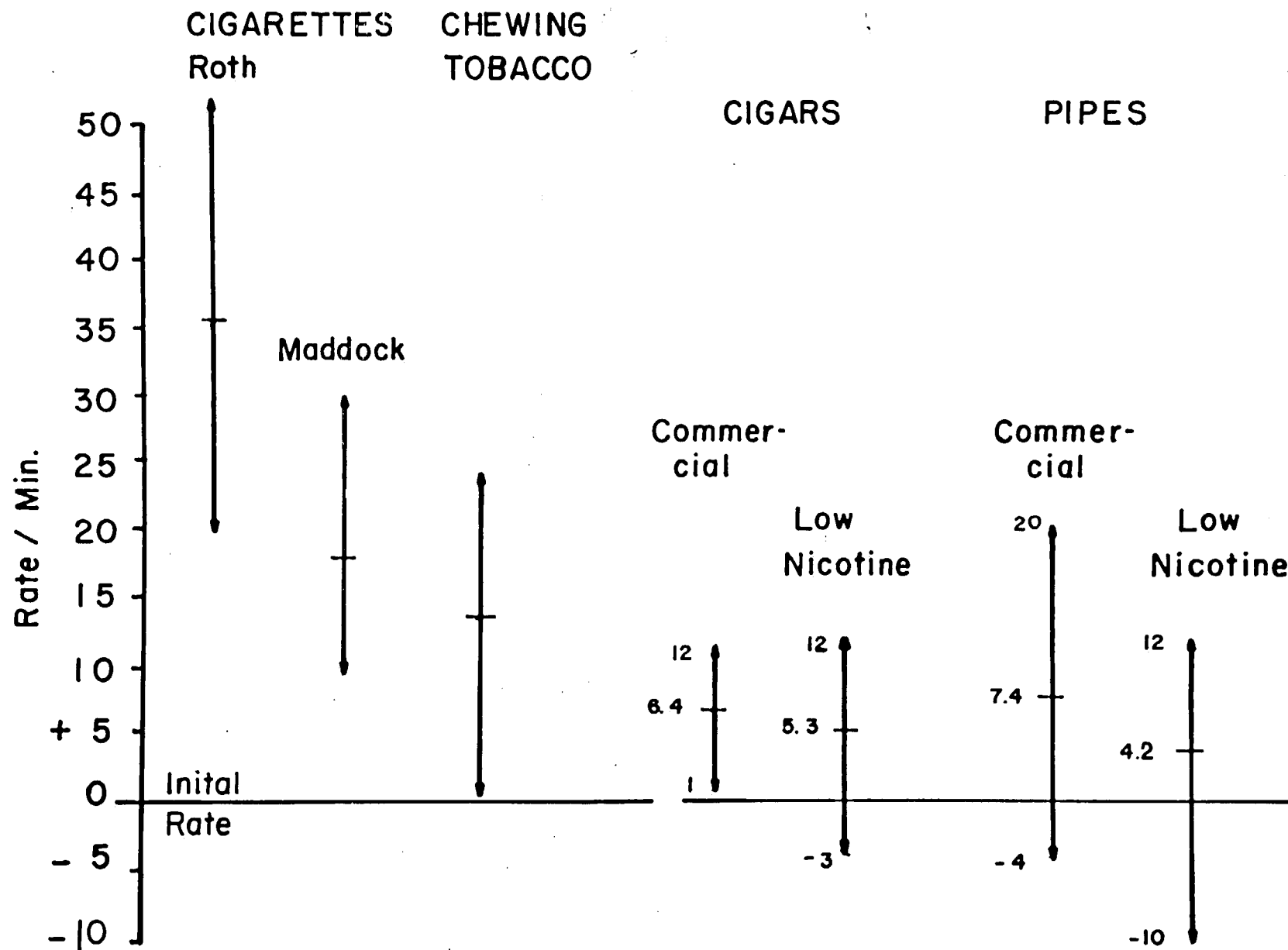
**21 Minutes**

**BALLISTOCARDIOGRAMS PIPE SMOKING**

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slide 2

# CHANGES IN PULSE RATE



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Slide III

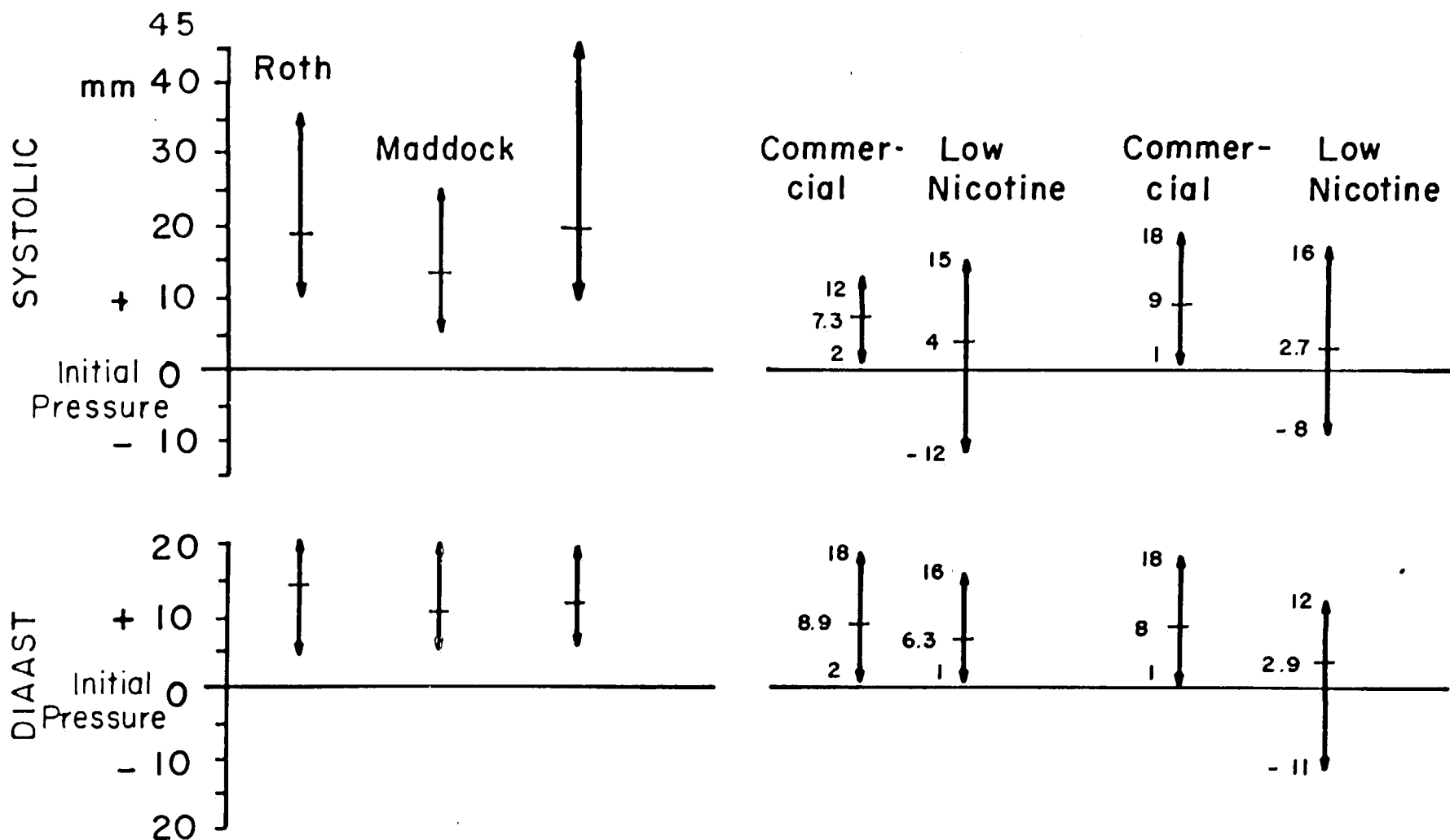
# CHANGES IN BLOOD PRESSURE

CIGARETTES

CHEWING  
TOBACCO

CIGARS

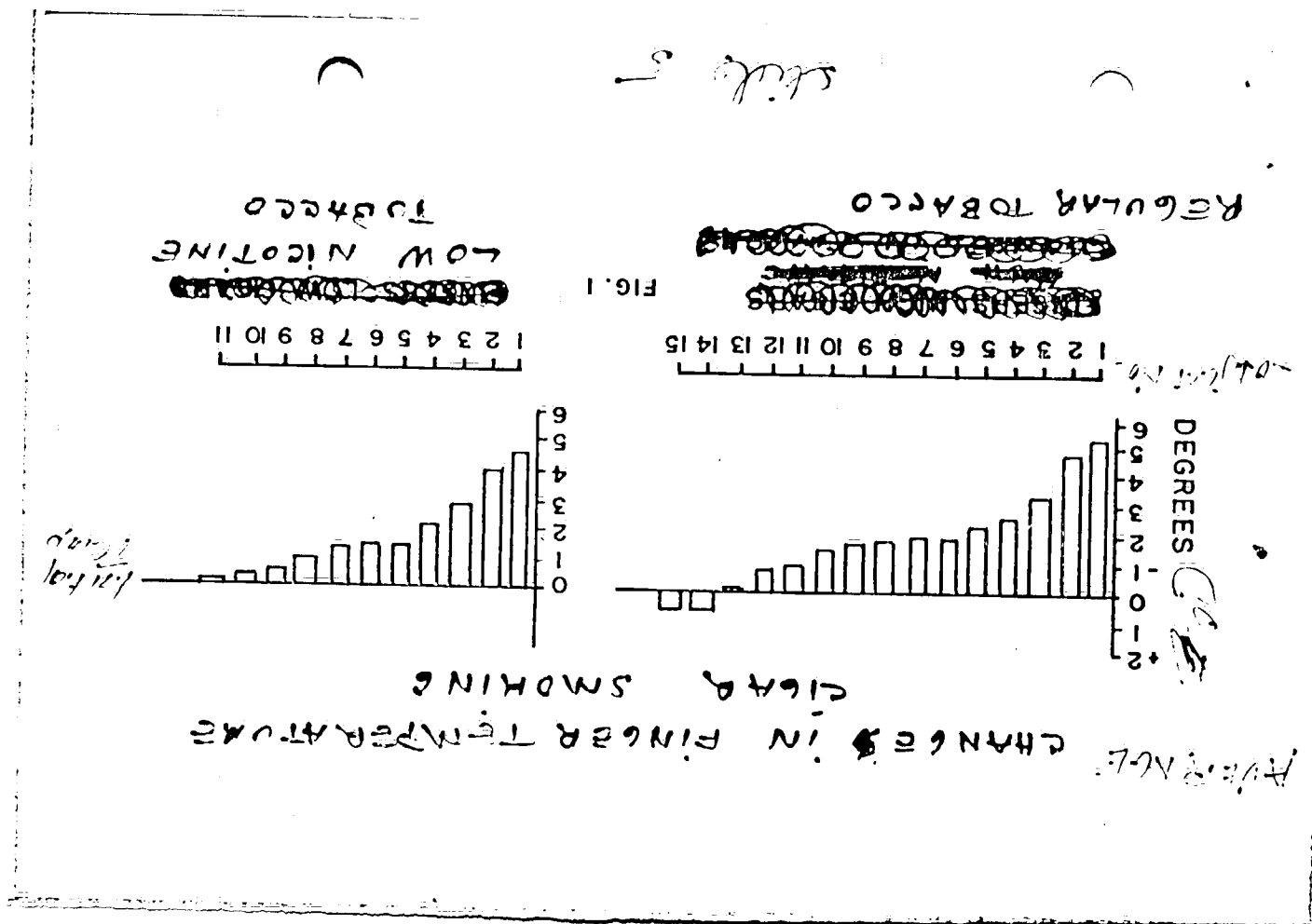
PIPES



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slide 12.

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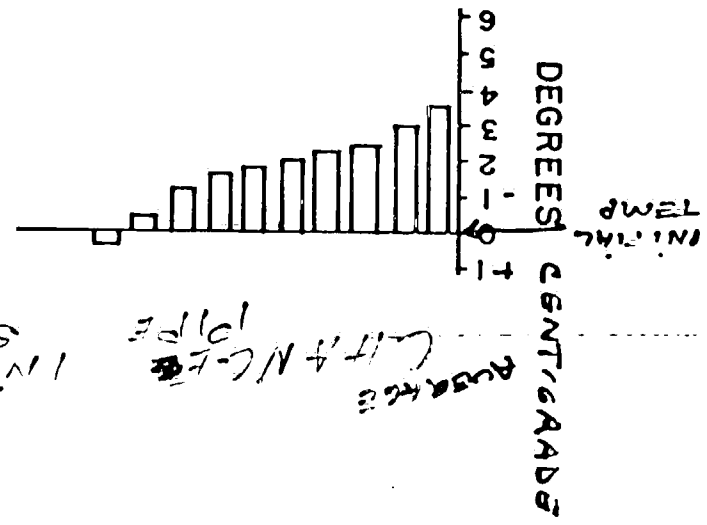
July 2

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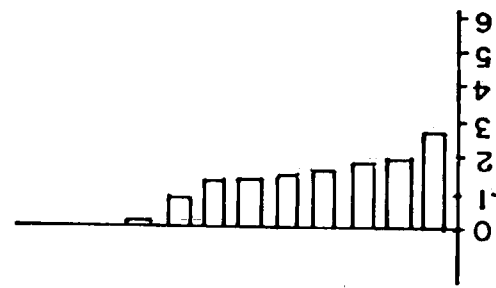
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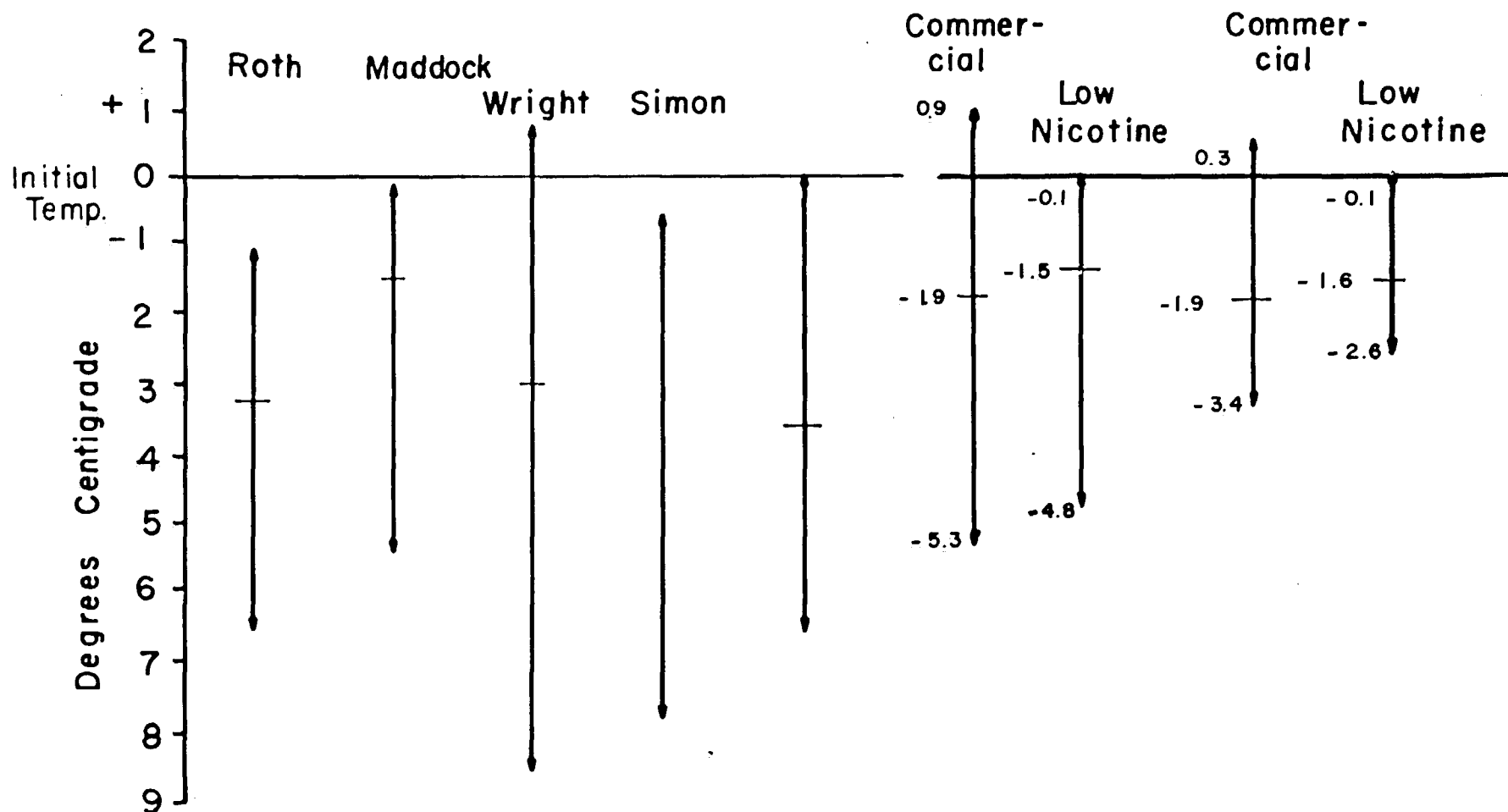
# CHANGES IN FINGER TEMPERATURES

CIGARETTES

CHEWING  
TOBACCO

CIGARS

PIPES



1003541199

slide 2

Committee:

TOBACCO INDUSTRY RESEARCH COMMITTEE

#263

Dr. Bing, Chm.  
Dr. Comroe  
Dr. Cattell

150 East Forty Second Street  
New York 17, N.Y.

Cf. #63  
Activated: 4/15/55  
and #148  
Activated: 7/1/57

Application for Research Grant

Date: February 3, 1960

1. Name of Investigator: Dr. David L. Simon and Dr. Arnold Iglauer
2. Title: Circulatory Effects of Snuff
3. Institution & Address: Cardiac Laboratory  
University of Cincinnati, Cincinnati General Hospital,  
Cincinnati 29, Ohio
4. Project or Subject: To round out our clinical studies on the circulatory effects of various forms of tobacco, we propose to study the effects of snuff on the heart and circulation in a manner similar to that used for the study of cigarettes, chewing tobacco, pipes and cigars.

5. Detailed Plan of Procedure: Twenty-five or more snuff users will be studied following the use of both regular snuff and a low nicotine snuff or a placebo and changes in the ballistocardiogram, skin temperatures, pulse rates and blood pressures will be observed.

Skin temperatures will be taken in a constant temperature room, as in our previous studies.  
Ballistocardiograms, pulse rates and blood pressures will be taken as in our previous studies.

We propose to study each individual on three separate days.

6. <u>Budget Plan:</u>	Salaries	\$ 3,200.
	Expendable Supplies	100.
	Permanent Equipment	-----
	Overhead	300.
	Other	-----
	Total	\$ 3,600.

1003541200

7. Anticipated Duration of Work: Six months to a year.
8. Facilities and Staff Available: Constant temperature room - Kettering Laboratory  
University of Cincinnati  
Cincinnati, Ohio  
  
Cardiac Laboratory  
Ballistocardiograph Laboratory  
Cincinnati General Hospital

Staff: Drs. Simon and Iglauer, Technicians, Research Fellows of the Cardiac Laboratory and one hired technician to help in the experiments.

9. Additional Requirements: None

Application (Dr. D. L. Simon #263)

February 3, 1960

10. Additional Information (Including relation of work to other projects and other sources of supply):

The subjects will be obtained from a large group of snuff users in this community. This will complete this series on our research into the effects of tobacco in its various forms on the heart and circulation.

/s/ David L. Simon  
Director of Project

/s/ R. M. Hoefer  
Business Officer

1003541201



TOBACCO INDUSTRY RESEARCH COMMITTEE  
150 EAST FORTY SECOND STREET NEW YORK 17, N.Y.

Application For Research Grant

#182

Date:

September 3rd, 1957

1. Name of Investigator:

Stanley C. Skoryna, M.D.

2. Title:

Research Director, Department of Experimental Surgery.

3. Institution:

& Address:

McGill University, Montreal, Canada.

4. Project or Subject:

Effects of Application of Tobacco Tars on Buccal Mucosa.

5. Detailed Plan of Procedure (Use reverse side if additional space is needed):

The objective of this project is to obtain data on the carcinogenic properties of the tobacco tars in relation to buccal mucosa. The differences in carcinogenic response between skin and mucous membranes appear to be of significance in tobacco-tar carcinogenesis. The hamster cheek pouch seems in many ways admirably suited for the studies outlined.

In preliminary investigations carried out in our Laboratories, it has been established that (1) tobacco tars solutions (including saturated solution) do not induce malignant or benign tumors of the mucous membrane of the hamster's cheek pouch, (2) 9, 10 dimethyl 1,2 benzanthrane is capable of inducing benign and malignant tumors under similar experimental conditions.

In the proposed program it is planned to carry out the following studies:

1. To enlarge and repeat the experiments with application of saturated solutions of tobacco tars and 9, 10 dimethyl 1, 2 benzanthrane.
2. To investigate possible significance of tobacco tars as initiating and promoting agents by a) application of saturated tobacco tars solution following a known initiating agent (benzanthrane), b) single tobacco tars application ~~following a known promoting agent (aroton oil)~~ followed by repeated application of a promoting agent (aroton oil).
3. To investigate the effects of cigar tars solution on buccal mucosa.
4. To investigate the effects of pipe tobacco tars on buccal mucosa.
5. To study the effects of tobacco tars on the growth of a hamster carcinoma transplanted into the cheek pouch.
6. To study histopathological and histochemical changes in relation to particular tissue components such as elastic tissue, reticulo-endothelial system and mucopolysaccharides. These studies would be carried during the next session not the current.

1003541202

6. Budget Plan:

Salaries	
Expendable Supplies	\$4,600
Permanent Equipment	3,200
Overhead	1,400
Other	600
Total	\$9,800

7. Anticipated Duration of Work:

8. Facilities and Staff Available: **2-3 years.**

The Experimental Surgical Laboratories are well equipped, also a Biochemical Laboratory. Various projects in experimental carcinogenesis have been carried out in these laboratories for the past 10 years.

9. Additional Requirements:

None

10. Additional Information (Including relation of work to other projects and other sources of supply):

Work is being carried out on various projects in chemical carcinogenesis, including carcinogenic activity of radioactive isotopes, under the auspices of the National Cancer Institute, National Research Council and Public Health Department.

Signature \_\_\_\_\_

Director of Project

/s/ Stanley C. Shoryna

\_\_\_\_\_  
Business Officer of the Institution

Vice President

1003541203

TOBACCO INDUSTRY RESEARCH COMMITTEE

#247

150 EAST FORTY SECOND STREET

New York 17, N.Y.

Application For Research Grant

Date: August 31, 1959

1. Name of Investigator: T. M. Sonneborn
2. Title: Distinguished Service Professor of Zoology
3. Institution  
& Address: Indiana University, 220 Jordan Hall  
Bloomington, Indiana
4. Project or  
Subject: Checking and extending the Stephano Paramecium test  
for carcinogenicity.
5. Detailed Plan of Procedure: Repeat the Stephano technique with genetically controlled strains of Paramecium aurelia, using first the same carcinogens and non-carcinogens already employed by Stephano.

If his results are confirmed, to extend the test to a considerable number of carcinogens and related non-carcinogenic compounds. For full analysis, there will be required not only fluorescent carcinogens and non-fluorescent non-carcinogens, but also--if they are available--fluorescent non-carcinogens and non-fluorescent carcinogens.

6. Budget Plan:

Salaries	\$ 4,000.00
Expendable Supplies	2,000.00
Permanent Equipment	-----
Overhead	960.00
Other	-----
Total	\$6,960.00
7. Anticipated Duration of Work: One year
8. Facilities and Staff Available: One technician, full-time, on this project. Part-time of workers in kitchen to supply clean glassware and solutions, etc.
9. Additional Requirements: Not at present foreseeable.

/s/ T. M. Sonneborn  
Director of Project

/s/ J. A. Franklin  
Vice President and Treasurer

1003541204

TOBACCO INDUSTRY RESEARCH COMMITTEE  
350 FIFTH AVENUE NEW YORK 1, N. Y.

6. Budget Plan

Salaries  
Frangible Supplies  
Application For Research Grant

#85  
2,000.00  
100.00  
Date: March 2, 1956

1. Name of Investigator: Sam Sorof, Ph.D.  
2. Anticipated Duration of Application of current grant from Tobacco Industry Research Committee for period ending September 30, 1956

2. Title: Associate Member  
3. Facilities and Staff Available

3. Institution & Address: The Institute for Cancer Research & Lankenau Hospital Research Inst.  
7701 Burholme Avenue, Fox Chase  
Philadelphia 11, Pa.

4. Project or Subject: Chemical and Physical Studies on the Tissue Proteins Involved in Chemical Carcinogenesis

9. Additional Requirements None

5. Detailed Plan of Procedure (Use reverse side if additional space is needed): Since May 9, 1955, the date of the application for the current financial grant from the Tobacco Industry Research Committee, our research has crystallized along lines summarized in the two submitted abstracts (1,2). Extensions of these studies are at present being directed toward the analysis, isolation, and characterization of a protein class ("slow  $h_2$ ") (1,2), which appears to be implicated in the carcinogenic process and which is present only to the extent of 1.1 per cent of all of the rat liver proteins. As a consequence, we have needed to grow large numbers of rats and feed them  $\alpha$ -azo dyes or the hepatocarcinogenic, 2-acetylaminofluorene, or control diets for 3 $\frac{1}{2}$  to 11 weeks. Each isolation experiment consumes approximately 85 such diet-fed rats, and yields 200-300 mg. of the fraction containing "slow  $h_2$ " proteins (1,2).

Because of these developments, which could not be foreseen when our application was made nearly 10 months ago, relatively large expenditures \* have been required since last October, when the grant became operative, for the processing of approximately sixteen hundred rats. In addition, funds have been appropriated for the increase of our rat housing and auxiliary facilities, such as cages, racks, carts, etc. As a consequence, our funds in the supply category of our current grant are now depleted. Since there is no other provision for the support of our project in the existing overall Institutional budget, request is hereby made for an additional \$2,000 for supplies for the remainder of the grant period, ending September 30, 1956.

REFERENCES

- (1) Sorof, S., Young, E. M., and Vogt, D. E. Proc. Am. Assoc. Cancer Research, 1956, in press  
Director of Project
- (2) Sorof, S., Ott, M. G., and Young, E. M., Federation Proc., 1956, in press.

\* A summary of these expenditures is appended in item 10.

1003541205

## 6. Budget Plan:

Salaries	\$ --
Expendable Supplies	2,000.00
Permanent Equipment	--
Overhead	160.00
Other	--
Total	\$2,160.00

7. Anticipated Duration of Work: Same as in application of current grant from Tobacco Industry Research Committee for period ending September 30, 1956

8. Facilities and Staff Available: ibid

The Institute for Cancer Research & Experimental Hospital Research Inst.  
7701 Locust Walk, Fox Chase  
Philadelphia 13, Pa.

Chemical and Physiological Studies of the Lipid Metabolism Involved in  
Carcinogenesis

9. Additional Requirements: None

10. Additional Information (including relation of work to other projects and other sources of supply): The date of the application for the current financial grant from the Tobacco Industry Research Committee, Summary of Expenditures on Current Grant, October 1, 1955 - February 28, 1956 (1, 2) Original budget called for supplies, amount going \$1,800.00 (3) Subsequently transferred from Equipment; \$200.00 (4) Subsequently transferred from Overhead; \$400.00 Total sum available as supplies, Oct. '55-Feb. '56 was \$2,400.00 Expenditures: General laboratory needs: \$1,100.00 Animal experimental needs: \$1,300.00 Animal foods: \$600 Housing, racks, etc., which could not be forecast when our application was made nearly a year ago, 300.00 Rats: 400.00 In addition, \$1,300.00 have been appropriated for the increase of our request for an additional \$2,000 for March, '56 - Sept. '56 for supplies is based on the best possible projection of our needs of recurring expenditures included in the above. Our request for the support of our project in the existing overall institutional budget, request is hereby made for an additional \$2,000 for supplies for the remainder of the grant period, ending September 30, 1956.

(1) Corof, S., Young, E. M., and Putney, G. Signature: s/ Sam Corof, 1956, in press. Director of Project  
(2) Corof, S., Ott, H. G., and Young, E. M., Federation Proc., 1956, in press.

\* A summary of these expenditures is included in item 10.

s/ H. D. Putney  
Business Officer of the Institution

1003541206



## 6. Budget Plan:

TOBACCO INDUSTRY RESEARCH CORPORATION  
350 FIFTH AVENUE NEW YORK 1, N.Y.

Salaries	\$10,710.00
Expendable Supplies	1,800.00
Apply Permanent Equipment	415.00
Overhead (6%)	1,068.00
Other (travel, service contract on analytical ultra-centrifuge)	430.00
Total	\$14,423.00

Date May 9, 1955

7. Anticipated Duration of Work: The work is part of a long term scientific project operating on a yearly fiscal basis. The present application represents our request for support during the period October 1, 1955 to September 30, 1956.

## 8. Facilities and Staff Available:

(a) Facilities: One analytical--preparative Spinco ultracentrifuge; one Klett Tiselius apparatus; one R Perkin--Elmer Tiselius apparatus; two walk-in cold rooms; one dark room; three laboratory rooms; two air-conditioned animal rooms; two preparative macroelectrophoresis cells; one electrophoresis convection apparatus; high speed centrifuges, Beckman DU and DK spectrophotometers, etc.

(b) Staff: Our research unit consists of:

1. Principal Investigator: Sam Sorof, Ph.D. (University of Wisconsin, 1949).
2. Research Assistant (full time): Dorothy E. Vogt, M.S. (Purdue University, 1955).
3. Research Assistant (full time): Emily M. Young, B.S. (University of Vermont, 1950).
4. Laboratory Helper (half time): Arthur Nelson

In addition, at this Institute, specialists in many biological and chemical disciplines are available for consultations and possible assistance. The Institute maintains breeding colonies of rats and a number of highly inbred mouse strains, various ascites tumors, excellent library, stock rooms, well equipped machine and repair shops with trained specialists.

#9 - Additional Requirements - NONE

## 10. Additional Information (including relation of work to other projects and other sources of supply):

(a) General: Certain dyes, when fed to rats or mice, produce hepatoma. The above outlined project represents the principal research effort of our unit. All work done by this group is directly involved in this undertaking.

The funds requested in this application represent the only provision for this project within the over-all Institutional budget, with the exception of the salary of the principal investigator.

## (b) Scientific

A number of aminoazo dyes have been found to produce specifically primary cancer of the livers of rats and mice. All other tested species and organs have been shown to be almost completely resistant to the carcinogenic action of these compounds. Administered by different means to different organs of different species. In other words, while the carcinogenicity with (Continued on added page) limited to the liver of rats and mice, and that is specific, the protein fraction which they attack may be of broad significance in the conversion of a normal cell into a malignant one.

We have isolated this presumably important protein fraction, in the expectation that chemical and biological study of the proteolytic activities involved will cast light on the basic mechanism of cancer. In addition, all proteins are representative of the Director of Projects in carcinogenesis. The work involved in the presumptive carcinogenic action of certain fractions of tobacco smoke.

/s/ H. D. Putiny (?)

Business Officer of the Institution

1003541208

May 9, 1955

## 10. Additional Information (including relation of work to other projects and other sources of supply):

b) Scientific (continued)

Drs. James A. and Elizabeth C. Miller of the University of Wisconsin have shown (1,2) that unknown derivatives of these ingested azo dyes unite only with liver proteins of the above species only. Furthermore, the more potent the carcinogen fed, the faster the accumulation of these protein-bound azo dyes in the liver. This combination of carcinogen with liver protein is a very firm one which can only be broken by complete degradation of the liver proteins, and thus far has only been formed by the intact animal. In vitro attempts to duplicate this combination have resulted in very weak attachments between proteins and azo dyes which can be easily split with various organic solvents. The Millers have reported that about 55 per cent of the protein-bound dyes are associated with the "soluble" proteins, as isolated by the present aqueous cytochemical techniques. These investigators also demonstrated the important fact that the azo-dye-induced liver tumor lacks these protein-bound carcinogens. This lack appears to be the result of the absence of such binding proteins, per se, or an inability to protein-bind azo dyes, since free azo dyes, uncombined with proteins, are present in these liver tumors. The Millers hypothesized that the in vivo formed derivatives of these azo dyes combine with certain proteins (enzymes) which are necessary for the control of the growth, but not for the life of liver cells. These proteins are thereby inactivated. In addition, these proteins are autolytic, i.e., they control the mechanism of their own protein reproduction. Hence, with each generation of liver cells there is less of these active growth-controlling enzymes. Eventually, a liver cell appears with less than the critical amount of these proteins, and as a consequence a series of irreversible, genetic reactions occur which result in the formation of the liver tumor cell.

Our findings, begun at the University of Wisconsin in collaborations with Dr. Philip P. Cohen and Drs. James A. and Elizabeth C. Miller, and continued at the Lankenau Hospital Research Institute and Institute for Cancer Research, have substantiated and extended this "protein deletion hypothesis." We found that a close pair of small electrophoretic components (labeled  $h_1$  and  $h_2$ ), consisting of the relatively basic proteins among the soluble proteins of rat liver, contain the bulk of the soluble protein-carcinogen derivatives during azo dye preneoplasia (3). In contrast to the preneoplastic liver and liver surrounding azo-dye-induced tumors, this " $h$ " fraction is almost absent in the tumors themselves and their distant metastases (4). The presence of protein-bound azo dyes in preneoplastic livers and liver surrounding azo-dye-induced tumors parallels the presence of " $h$ " proteins therein, while the lack of " $h$ " proteins in tumors parallels the absence of protein-bound azo dyes in these tumors. The reduction in size of the " $h$ " component appears to be associated with neoplasia itself, rather than rate of growth, per se, of the

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## 10. Additional Information Etc.

b) Scientific (continued)

tumors, since regenerating liver and liver of the fasted rat both closely resemble electrophoretically the liver of the normal stock rat (5). Interestingly, a variety of unrelated tumors (e.g., 2-acetylaminofluorene-induced liver tumor, a number of transplanted tumors, one human tumor thus far) electrophoretically exhibit a similar deficiency in the amount of the "h" proteins (4). In addition, this similarity exists among a number of tumors investigated by others (e.g., fibrosarcomas induced by methycholanthrene and benzpyrene (6).)

At the Lankenau Hospital Research Institute and Institute for Cancer Research, we have been extending our study of these "h" proteins. Using a new technique (7), we have isolated a major ultracentrifugal class of soluble liver proteins and found that the major share of the soluble protein-bound dyes is present therein (8). In addition, new methods and principles of electrophoretic fractionation have been developed (9,10) which have been applied to the isolation of the "h" proteins. Essentially by these techniques in unpublished studies, we have isolated the  $h_1$  and  $h_2$  proteins. Interestingly, both  $h_1$  and  $h_2$  have protein-bound dyes. This may be interpreted to fit the observed fact that both types of proteins are almost lacking in the azo-dye-induced tumor. Some physical and chemical properties of these proteins have been investigated. These findings are now being prepared for publication (11). We plan to extend our physical and chemical studies of these proteins obtained from rats fed control and various azo dyes of differing carcinogenicities in order to attempt to shed additional light on the mechanism of azo dye carcinogenesis, in particular, and the nature of the malignant transformation, in ~~general~~ general. Thus, if these cell proteins are representative of specific targets in ~~carcinogenesis~~ carcinogenesis, they may likewise be involved in the presumptive carcinogenic action of certain fractions of tobacco smoke.

References

- (1) Miller, E. C. and Miller, J. A. Cancer Research, 7, 468 (1947).
- (2) Miller, E. C., Miller, J. A., Sapp, R. W. and Weber, G. M. Cancer Research, 9, 336 (1949).
- (3) Sorof, S., Cohen, P. P., Miller, E. C., Miller, J. A. Cancer Research, 11, 383 (1951).
- (4) Sorof, S. and Cohen, P. P. Cancer Research, 11, 376 (1951).

1003541210

References

- (5) Sorof, S., Claus, B. and Cohen, P. P. Cancer Research, 11, 873 (1951).
- (6) Barry, G. T. Cancer Research, 10, 694 (1950).
- (7) Sorof, S. J. Am. Chem. Soc., 75, 5443 (1953).
- (8) Sorof, S., Golder, R. H., Ott, M. G. Cancer Research, 14, 190 (1954).
- ~~(9) Sorof, S., Ott, M. G., Young, E. M. Arch. Biochem. Biophys., in press.~~
- (9) Sorof, S. and Ott, M. G. J. Am. Chem. Soc., 76, 4740 (1954).
- (10) Sorof, S., Ott, M. G., Young, E. M. Arch. Biochem. Biophys., in press.
- (11) Sorof, S., Ott, M. G., Young, E. M. to be published.

1003541211

**CONFIDENTIAL**

TIRC Grant #85

Progress Report #1

Dr. Sam Sorof

February 1956

The Institute for Cancer Research  
and Lankenau Hospital Research Institute

"Chemical and Physical Studies on the Tissue Proteins  
Involved in Chemical Carcinogenesis"

Increase of Certain Soluble Liver Proteins Associated with Dye Binding  
during Aminoazo Dye Hepatocarcinogenesis in the Rat.

Sam Sorof, Emily M. Young and Dorothy E. Vogt

Protein-bound dyes are present almost specifically in the livers of rats fed hepatocarcinogenic azo dyes and are absent in the induced tumors (Miller and Miller, Cancer Research, 7:468, 1947). The soluble bound dyes are relatively localized in an electrophoretic class ("h") of proteins (Sorof, Cohen, Miller and Miller, Cancer Research, 11:383, 1951). These proteins are greatly reduced in quantity in the azo dye induced liver tumor (Sorof and Cohen, Cancer Research, 11:376, 1951).

Lankenau-Wistar rats of both sexes were fed ad libitum 0.06% 4-dimethylaminoazobenzene (DAB) for 32 days, or 0.057% of its 3'-methyl derivative (3'-Me-DAB) for 18-21 days, incorporated into diet #3 of Miller and Miller. Control rats were similarly fed the same diet without dye. Using the single sucrose density gradient as a barrier to convection (J. Am. Chem. Soc., 76:4740, 1954), the resolution of the "h" proteins has now been increased 27-fold, thereby revealing six distinct sub-components. Our findings to date indicate that the relative quantities of one of these "h" components from rats fed DAB or 3'-Me-DAB are respectively 76 and 62 per cent greater than their controls. By comparison, 3-days regenerated livers exhibit only a 10-20 per cent increase in this component. Evidence indicates that in the case of these dye fed rats this component represents azoproteins. Speculatively, and in possible agreement with the Miller "protein-deletion" hypothesis (l.c.), the observed preneoplastic increase of this sub-class of "h" proteins may constitute an ultimately unsuccessful compensatory response against the effects of their binding of azo dyes.

Reprinted from Proceed. AACR, 1956

1003541212

CONFIDENTIAL

TIRC Grant #85

Progress Report #2

Dr. Sam Sorof

March 1956

The Institute for Cancer Research  
and Lankenau Hospital Research Inst.

"Chemical and Physical Studies on the Tissue  
Proteins Involved in Chemical Carcinogenesis"

Partial Purification and Preliminary Analyses of Certain Soluble Azoproteins from Livers of Rats Fed 3'-Methyl-4-dimethylaminoazobenzene. Sam Sorof, Marilyn G. Ott, and Emily M. Young.

Previous studies appear to indicate the involvement of the soluble liver "h" azoproteins in aminoazo dye hepatocarcinogenesis in the rat through the hypothetical "protein deletion" mechanism of Miller and Miller (Adv. in Cancer Research 1:339, 1953). Adult Lankenau-Wistar rats of both sexes were fed ad libitum for 18-21 days diet #3 (Miller and Miller) including 0.057% 3'-methyl-4-dimethylaminoazobenzene and 1.0 mg. riboflavin/kg. Control rats were fed the same diet without dye. Using free boundary electrophoresis with a convection barrier (J. Am. Chem. Soc. 76:4740, 1954), 100-300 mg. quantities of the following two fractions have been isolated in each separation from six resolved "h" sub-components: (1) 100% "h<sub>3</sub>" (2) three "h" sub-classes: 57% "h<sub>3</sub>"; 30% "slow h<sub>2</sub>"; 13% "middle h<sub>2</sub>". These represent 7.3%, 3.4% and 3.5%, respectively, of the soluble liver proteins of the dye fed rats. The "h<sub>3</sub>" proteins contain 14% of all the soluble bound dyes. Assuming the absence of bound dyes with the minor constituent ("middle h<sub>2</sub>") in Fraction 2, 42% of all the soluble bound dyes are with the same sub-class ("slow h<sub>2</sub>") which greatly increases during azo dye preneoplasia (Proc. Am. Assoc. Cancer Research, in press, 1956). The "h" proteins of Fraction 2 from control or dye fed rats do not contain significant amounts of non-dialyzable nucleic acids or riboflavin. This is of interest considering the roles of nucleic acids in growth and the inhibitory effect of dietary riboflavin on certain azo carcinogens.

Reprinted from Fed. Proceed., 1956

1003541213

TIRC Grant #85

Progress Report #3

Dr. Sam Sorof

The Institute for Cancer Research  
and Lankenau Hospital Research Inst.Oct. 1, 1955 to  
Aug. 1, 1956

"Chemical and Physical Studies on the Tissue  
Proteins Involved in Chemical Carcinogenesis"

Certain azo dyes, when fed to rats or mice, produce liver tumors. These same dyes have been found to combine with only the liver proteins of only those species. So far, no other species, nor organ other than liver, have been found to be similarly affected by these particular dyes. The dyes combine metabolically with a protein fraction (h-proteins) of rat liver, and the liver tumors produced by the dye contain neither the dye nor essentially this h-protein fraction. A particular azo dye (2-methyl-4-dimethylaminoazobenzene) is sufficiently similar to the carcinogenic azo dyes in structure so that although it is non-carcinogenic, its derivatives bind to liver proteins. However, of critical importance is the fact that all known azo hepatocarcinogens bind to liver proteins.

In the past year, a small subfraction of h-proteins, the slow-h<sub>2</sub> proteins, have been found to contain relatively large amounts of bound dyes derived from three carcinogenic dyes, as well as from the above non-carcinogenic dye. However, thus far, only the feeding of the carcinogenic dyes has greatly increased the relative amount of the slow-h<sub>2</sub> proteins. The above structurally-close non-carcinogen has not. The known increased rate of growth, per se, associated with azo dye preneoplasia, as well as decreased dietary intake associated with azo dye feeding, are both excluded as possible causes of the elevated amounts of slow-h<sub>2</sub> proteins. Up to the present time, the only reasonable correlation with the increase of slow-h<sub>2</sub> proteins appears to be the necessity of protein-binding and carcinogenicity.

As a purely speculative working hypothesis, we wonder whether the observed preneoplastic increase of the slow-h<sub>2</sub> proteins may constitute a specific response against the effects of their binding of carcinogenic azo dye derivatives, as opposed to those from non-carcinogenic azo dyes. If so, this elevation of slow-h<sub>2</sub> proteins may be an ultimately unsuccessful attempt to compensate against their dye binding, with the consequent already demonstrated loss of h-proteins in the azo dye-induced tumor. Therefore, we further wonder whether possibly the slow-h<sub>2</sub> proteins may be the specific target proteins in azo dye carcinogenesis.

1003541214

Part IPartial Purification and Preliminary Analyses of Certain Soluble Azoproteins from Livers of Rats Fed Aminoazo Dyes.

by

Sam Sorof, Marilyn G. Ott and Emily Young

Miller and Miller have demonstrated that the livers of rats fed carcinogenic azo dyes or certain seemingly exceptional non-carcinogenic azo dyes contain protein-bound dyes (1,2). However, protein-bound dyes are absent in the resulting induced liver tumor. Price, Miller and Miller (3) found that over one-half of these dyes are bound to soluble liver proteins. Seemingly in parallel with these findings, Sorof, Cohen, Miller and Miller demonstrated that the liver proteins of a small pair of electrophoretic components,  $h_1$  and  $h_2$ , contain the bulk of these soluble bound azo dyes (4). In addition, Sorof and Cohen (5) reported that compared to normal, preneoplastic, or non-neoplastic rat liver, the amount of these h-proteins is markedly reduced in dye-induced liver tumors. These and other findings appear to be compatible with the "protein deletion" hypothesis of azo dye carcinogenesis of Miller and Miller (1,2).

Only the final phases of this study (6) were completed during the covered period. This investigation dealt with the electrophoretic resolution of the h-proteins into six very small subclasses; the isolation of two such partially purified h-subfractions; the concentration of protein-bound azo dyes therein; and the absence of significant amounts of either nucleic acid or riboflavin in such electrophoretically isolated and dialyzed h-proteins.

Adult rats of both sexes were fed *ad libitum* either 0.06% of the parent carcinogen, 4-dimethylaminoazobenzene (DAB), for  $4\frac{1}{2}$  to 5 weeks; or 0.058% of the more potent carcinogen, the 3'-methyl derivative (3'-Me-DAB), for 18 to 21 days; or 0.064% of the very weak carcinogen, 4'-Me-DAB, for 11 weeks; or 0.058% of the non-carcinogen, 2-Me-DAB, for 11 weeks incorporated into the 12% casein diet #3 of Miller and Miller (7), containing 1.0 mg. riboflavin/kg. As shown by Miller and Miller (1,2), the liver contains maximum quantities of the corresponding protein-bound dyes resulting from such feedings at these times. Control rats were similarly fed the same diet without dye for equal periods of times.

In the preparation of the soluble proteins of rat liver, all rat livers were perfused with cold homogenizing buffer and then homogenized in a Potter-Elvehjem homogenizer in 0.08 M sodium phosphate, pH 7.8, containing 0.075 M sodium chloride. This homogenate was ultracentrifuged at 115,000 g for one hour. Use of concentrated protein solutions, large electrophoresis cells of long optical depth, together with long optical lever arm have permitted the detection of very small electrophoretic components which have been revealed by the very high separating power of convection barrier electrophoresis, a method originated in our laboratory (8). In this technique, a single sucrose density gradient placed at the bottom of a column of protein solution effectively isolates disrupting convection gradients from the separating protein solution above, overcoming thereby the hitherto practical limit in the electrophoresis of slowly migrating components in natural protein mixtures.

Of particular practical importance is the finding that chloride ion apparently specifically prevents the irreversible insolubilization of h-proteins during an early stage of electrophoretic fractionation. Hence, almost all buffers coming in contact with h-proteins in our experiments have contained chloride ion.

By these means, the h-proteins have been electrophoretically expanded 27 times that formerly reported (9). Thus, the  $h_1$ -proteins have been resolved into two components; the  $h_2$  into three; and the presence shown of another component,  $h_3$ , previously hidden in the salt boundary. Relatively minute components have thus been revealed with a very high degree of resolution between components differing by as little as 0.13 of a mobility unit. Hence, for example, the slow- $h_2$  proteins of the above DAB or 3'-Me-DAB fed rats represent 1.0% of all the liver proteins.

The following two fractions of h-proteins from rats fed the 3'-Me-DAB diet were isolated: (a) the  $h_3$  region, (b) the slow  $h_2$  + middle  $h_2$  region. These were then analyzed electrophoretically in sodium veronal, pH 8.6, 0.1M. Since the descending proteins denature due to the absence of chloride, only ascending patterns were so analyzed. Figure I shows the analyses of isolated  $h_3$ -proteins. Only after prolonged electrophoresis does an asymmetry appear, indicative of heterogeneity. However, electrophoreses at lower pH and ultracentrifugal analyses each reveal the presence of three types. Figure II shows similar analyses of the other isolated fraction, consisting of 57%  $h_3$ , 30%  $h_2$ -slow, and 13%  $h_2$ -middle.

Assuming the absence of protein-bound dye with the middle- $h_2$  proteins, present only as 13% of this fraction, direct protein bound dye analyses indicate in the case of 3'-Me-DAB fed rats that the  $h_3$ -proteins, which represent 6.8% of all the soluble proteins of rat liver, contain approximately 12% of the soluble protein-bound dyes. Likewise, the slow- $h_2$  proteins, which are only 1.9% of all the soluble proteins of these azo dye fed rats contain approximately 24% of all the protein-bound dyes. Similar results have been obtained with the other carcinogens, DAB and 4'-Me-DAB, as well as with the protein-binding non-carcinogen, 2-Me-DAB (see Part II).

Through the original studies of Kensler and co-workers (10), and many thereafter, dietary riboflavin is known to inhibit certain azo carcinogens. In addition, the nucleic acids have been implicated in protein synthesis, growth, and heredity. For these reasons, it appeared of interest to determine whether the isolated soluble h-proteins from azo dye or control fed rats might contain flavo- or nucleoproteins. Since these h-proteins were isolated by electrophoresis and subsequent dialyses in the cold against pH 7.4 buffer, followed by distilled water, possibly the preparative electrophoresis and the dialyses may have removed any flavin or nucleic acid present, if they dissociate under these conditions. However, within this limitation, it can be said that as isolated the  $h_3$ - and  $h_2$ -slow proteins from control or 3'-Me-DAB fed rats do not contain significant amounts of either riboflavin or nucleic acid. Table I shows that the RNA content analyzed to be only 0.02% and the DNA content found was only 0.07%. Likewise, as presented in Table II, fluorimetric analysis for riboflavin, based on the assumed usually found 1 mole of flavin per 70,000 grams of protein, revealed the extremely small maximum probable flavoprotein content in these isolated h-proteins of less than 0.15% for rats fed 3'-Me-DAB, and 0.02% for their control.

1003541216

In summary, by original techniques the h-proteins, previously found to contain the bulk of soluble azoproteins after azo-carcinogen feeding and found to be sharply reduced in the azo dye-induced tumor, have been electrophoretically resolved into six subclasses. Two partially purified h-subfractions have thus been isolated and shown to contain protein-bound azo dyes. Further, such isolated and dialyzed h-proteins from control or dye fed rats do not contain significant amounts of nucleic acid or riboflavin. This is of interest considering the roles of the nucleic acids in growth and the inhibitory effect of dietary riboflavin on certain azo carcinogens.

1003541217



## Part II

Increase of Certain Soluble Liver Proteins  
Associated with Dye Binding during Aminoazo  
Dye Hepatocarcinogenesis in the Rat

by

Sam Sorof, Emily M. Young and Dorothy E. Knospe

The second phase (12) of our work of the past year has dealt with the behavior of the proteins of one, small sub-class of these same soluble rat liver h-proteins, the slow-h<sub>2</sub> proteins. These proteins represent only 1.0 to 1.9% of all soluble rat liver proteins, exact quantity depending upon experimental conditions to be described. In other terms, the slow-h<sub>2</sub> proteins represent only between 0.5 to 1.0% of all proteins of rat liver. Evidences appear to be compatible with the possible direct involvement of this small group of proteins, the "slow-h<sub>2</sub>" proteins, in azo dye carcinogenesis.

The azo carcinogenic, azo non-carcinogenic, and control diets were fed to rats exactly as described in Part I of this Progress Report. The soluble proteins of liver were then prepared and analyzed electrophoretically by methods also summarized there.

The soluble azoproteins absorb liver maximally at wavelength 405 m $\mu$ . Hence, this property may be used to determine the location of azoproteins of separated components in the electrophoresis cell. Figure III shows the absence of light absorption of wavelength greater than 530 m $\mu$  by the h-proteins of rats fed DAB or control diets. However, using light of wavelength 390 to 450 m $\mu$ , the separated azoproteins from DAB fed rats absorb the light, in contrast to the control diet pattern, shown in juxtaposition. The beginning of the light absorption corresponds to the beginning of the slow-h<sub>2</sub> proteins. The lack of absorption by the h<sub>3</sub>-proteins appears to indicate the relative lack of azoproteins with this component. Similar experiment has been done with the other tested dyes. These experiments do not offer information about the possible azoprotein content of the other adjacent components, since the slow-h<sub>2</sub> proteins are present throughout the remainder of the cell. In any event, the slow-h<sub>2</sub> proteins are thus shown to contain azoproteins. By direct analyses of isolated fractions in the former study (6), the slow-h<sub>2</sub> proteins account for 24% of all the soluble protein-bound dyes of rats fed the more potent carcinogen, 3'-Me-DAB, for 2½ to 3 weeks. Similarly, in the case of rats fed the parent carcinogen, DAB, for 4½ to 5 weeks, the slow-h<sub>2</sub> proteins contain 22% of all of the soluble protein-bound dyes; while the corresponding value for the extremely weak carcinogen, 4'-Me-DAB, fed 11 weeks is 24%. Likewise, in the case of the rats fed the non-carcinogen, 2-Me-DAB, for 11 weeks, the slow-h<sub>2</sub> proteins contain 19% of all soluble protein-bound dyes.

These slow-h<sub>2</sub> proteins, which contain azoproteins, have also been observed to exhibit an interesting response to azo dye feeding. Figure IV illustrates the effect on the amount of slow-h<sub>2</sub> proteins caused by the feeding of 3'-Me-DAB for 18-21 days, when the resultant protein-bound dyes are at their maximum level in liver. The average increase in the slow-h<sub>2</sub> proteins from such 3'-Me-DAB fed rats has been 48%, compared to the corresponding control proteins.

1003541218

In order to determine the cause of this observed increase of the slow-h<sub>2</sub> proteins, a series of experiments have been performed the results of which are summarized below. Compared to the 48% increase for the twice as potent carcinogen, 3'-Me-DAB, the parent carcinogen tested, DAB, caused an increase of 60% after feeding 4½ to 5 weeks, its corresponding peak time of liver protein-bound dyes. Similarly, the very weak carcinogen, 4'-Me-DAB, caused a 53% increase of slow-h<sub>2</sub> proteins after a feeding of 11 weeks. However, the non-carcinogen, 2-Me-DAB, compared to its control, also fed for the binding peak time (11 weeks), thus far has caused no increase in slow-h<sub>2</sub> proteins. This is so despite the fact that 2-Me-DAB is structurally so similar to the azo carcinogens that it is capable of forming relatively large amounts of hepatic protein-bound dyes (3), including those bound to slow-h<sub>2</sub> proteins.

That this increase in the amount of slow-h<sub>2</sub> proteins is not due to the known stimulated growth, per se, associated with carcinogenic azo dye feeding is seen in the control-like level of these proteins from the 3 days' regenerated rat liver. Likewise, this increase cannot be caused by the decreased dietary intake of azo dye feeding, since the 2-Me-DAB fed rats, which eat less than those on the other tested dyes, did not show any increase of slow-h<sub>2</sub> proteins. Similarly, this increase cannot be due to protein binding only, because the 2-Me-DAB causes relatively large amounts of liver protein-bound dyes, including those bound to slow-h<sub>2</sub> proteins. Up to the present time, at least, the only reasonable correlation with the increase of the slow-h<sub>2</sub> proteins appears to be the necessity for both protein-binding and carcinogenicity. As a purely speculative working hypothesis, which we of course are not prepared to defend, we wonder whether the observed preneoplastic increase of the slow-h<sub>2</sub> proteins may constitute a specific response against the effects of their binding of carcinogenic azo dye derivatives, as opposed to those from non-carcinogenic azo dyes. If so, this response of these proteins may be an ultimately unsuccessful attempt to compensate against their dye binding, with the consequent already demonstrated loss of h-proteins in the azo dye induced tumor (5). Therefore, we further wonder whether possibly the slow-h<sub>2</sub> proteins may be the specific target liver proteins in azo dye hepatocarcinogenesis.

In summary, a small sub-class of h-proteins, the slow-h<sub>2</sub> proteins, have been shown to contain a relatively large share of soluble azoproteins resulting from ingested carcinogenic or non-carcinogenic azo dyes. Only the tested azo carcinogens, DAB, 3'-Me-DAB, and 4'-Me-DAB, induce a relatively large increase in the amount of slow-h<sub>2</sub> proteins. The structurally-close non-carcinogen, 2-Me-DAB, although capable of also forming dyes bound to these slow-h<sub>2</sub> proteins, has thus far not been found to cause such an increase in these slow-h<sub>2</sub> proteins. Thus far, the only apparent correlation with this increase appears to be the necessity for both protein-binding to these slow-h<sub>2</sub> proteins and carcinogenicity of ingested azo dyes.

1003541219

References

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- (2) Miller, E. C.; Miller, J. A.; Sapp, R. W.; and Weber, G. W. Cancer Research, 9, 336 (1949).
- (3) Price, J. M.; Miller, E. C.; and Miller, J. A. J. Biol. Chem. 173, 345 (1948). Price, J. M. et al., Cancer Research, 9, 398 (1949). Price, M. M. et al., Ibid., 10, 18 (1950).
- (4) Sorof, S.; Cohen, P. P.; Miller, E. C.; and Miller, J. A. Cancer Research, 11, 383 (1951).
- (5) Sorof, S. and Cohen, P. P. Cancer Research, 11, 376 (1951).
- (6) Sorof, S.; Ott, M. G.; and Young, E. M. Federation Proc., 15, 358 (1956).
- (7) Miller, E. C.; Miller, J. A.; Kline, B. E.; and Rusch, H. P. J. Exper. Med., 88, 89 (1948).
- (8) Sorof, S. and Ott, M. G. J. Am. Chem. Soc., 76, 4740 (1954).
- (9) Sorof, S. and Cohen, P. P. J. Biol. Chem., 190, 311 (1951).
- (10) Kensler, C. J.; Sugiura, K.; Young, N. F.; Halter, C. R.; and Rhoads, C. P. Science, 93, 308 (1941).
- (11) Singer, T. P. and Kearney, E. B. in "The Proteins" (H. Neurath and K. Bailey, ed.) Vol. IIA, p. 222. Academic Press Inc., New York, 1954.
- (12) Sorof, S.; Young, E. M.; and Vogt, D. E. Proc. Am. Assoc. Cancer Research, 2, 148 (1956).

1003541220

Table I

Diet	protein equivalent color-developed, mg.	$\frac{\gamma \text{ RNA-P}}{\text{mg. N}}$	$\frac{\text{RNA}}{\text{protein}}\%$	$\frac{\gamma \text{ DNA-P}}{\text{mg. N}}$	$\frac{\text{DNA}}{\text{protein}}\%$
3'-Me-DAB	15.5	0.2	0.3	undetectable	
3'-Me-DAB	16.2	undetectable		—	—
3'-Me-DAB	17.3	0.2	0.03	0.9	0.14
avg.		0.1	0.02	0.5	0.07
Control	19.8	0.1	0.02	0.5	0.08
Control	15.8	0.1	0.02	0.4	0.06
avg.		0.1	0.02	0.5	0.07

Nucleic acid analyses of isolated "h<sub>3</sub> + slow-h<sub>2</sub> + middle h<sub>2</sub>" fraction from rats fed 3'-Me-DAB or control diets for 18-21 days.

1003541221

Table II

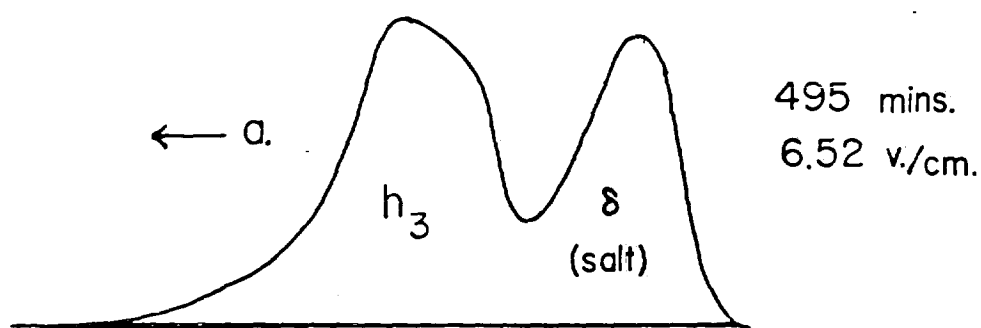
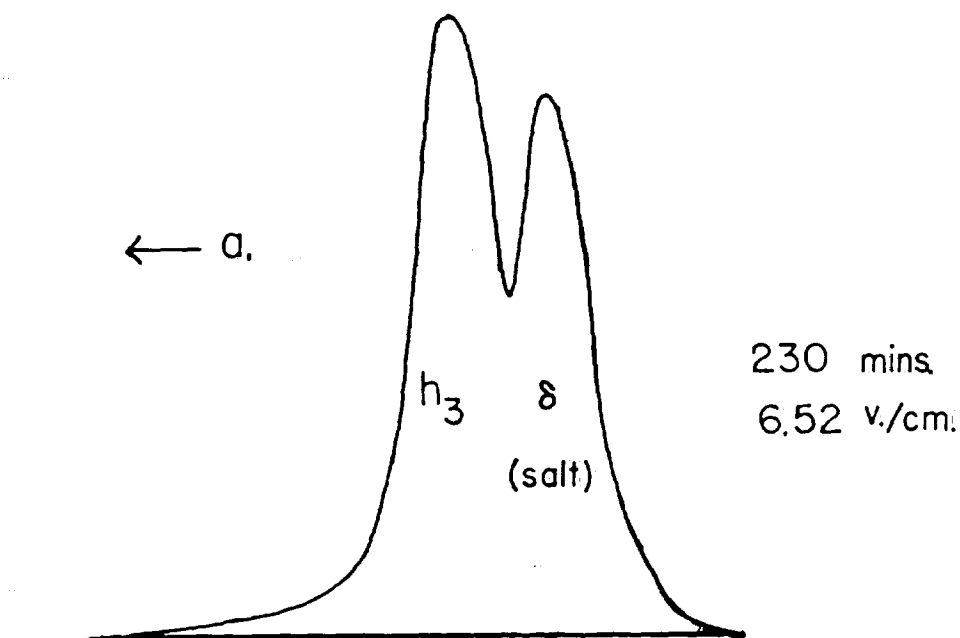
Diet	Wt. of protein mg.	Riboflavin Content $\delta$ /gm.	"Flavoprotein" Content* %
3'-Me-DAB	171	< 10	} < 0.15
3'-Me-DAB	233	< 5	
3'-Me-DAB Control	348	1	} 0.02
3'-Me-DAB Control	245	ca. 0.7	

\* Based on average 1 mole of flavin per 70,000 grams of protein (11).

Riboflavin and "flavoprotein" contents of dialyzed azo- or control "h<sub>3</sub> + slow h<sub>2</sub> + middle h<sub>2</sub>" protein fraction from rats fed 3'-Me-DAB or control diets, respectively, for 18-21 days.

1003541222

Figure I.

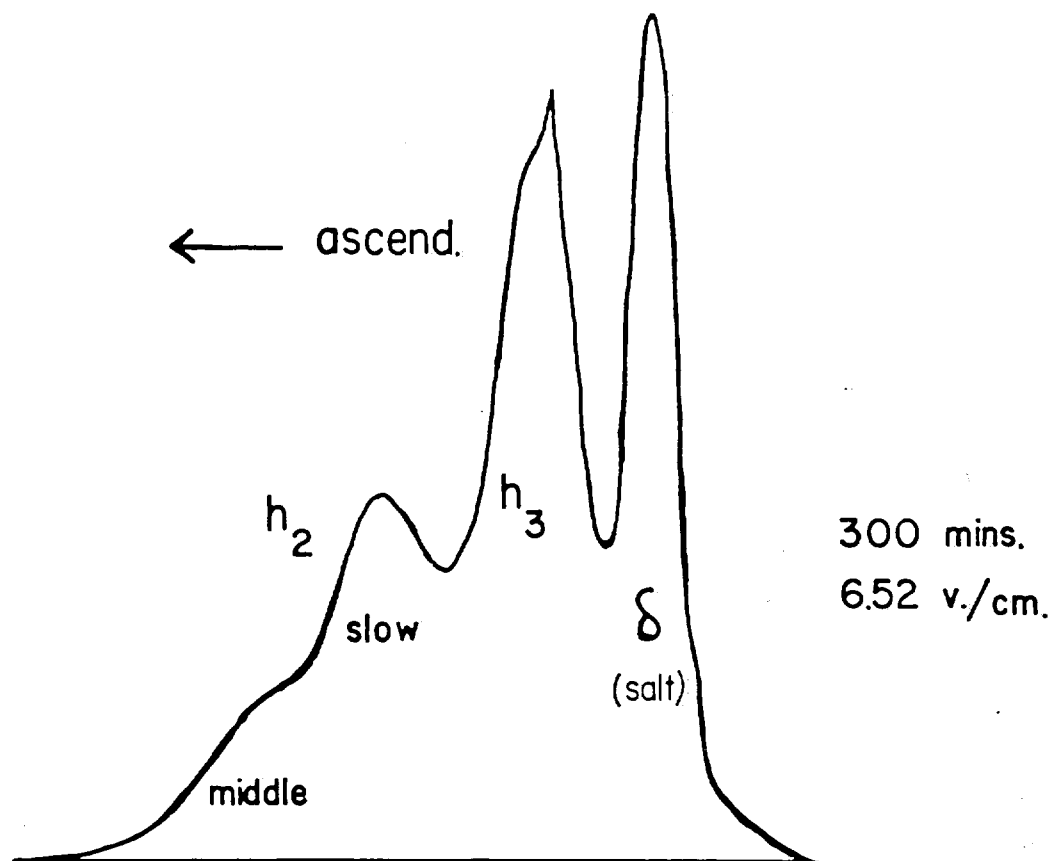


ELECTROPHORETIC DIAGRAMS OF ISOLATED  
 $h_3$  PROTEINS.

3'-Me-DAB CONTROL - 3 WKS.; NaV, 0.1  $\mu$ , pH 8.6;  
1.3 % PROTEIN

1003541223

Figure 11.



ELECTROPHORETIC DIAGRAM OF ISOLATED  $h_2$  &  $h_3$  PROTEINS.

DAB CONTROL-4½ WKS.; 3.1% PROTEIN; NaV, 0.1μ, pH 8.6

1003541224

DAB

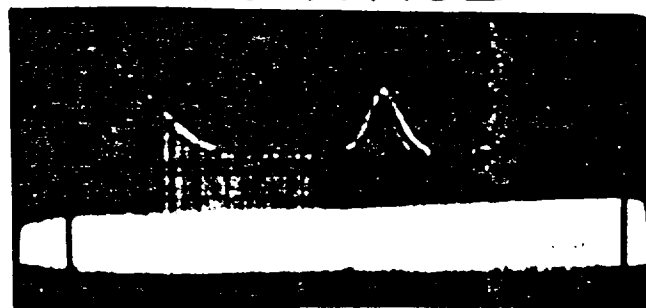


> 530 mμ

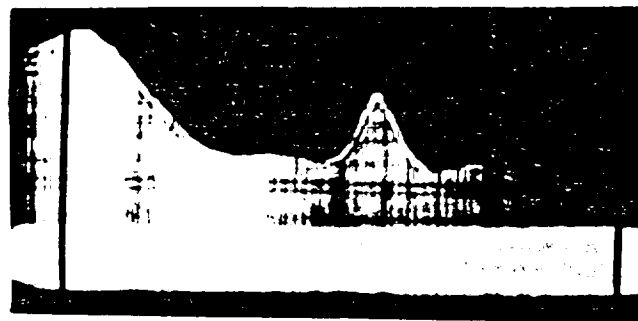


420 (390-450) mμ

CONTROL



> 530 mμ



420 (390-450) mμ

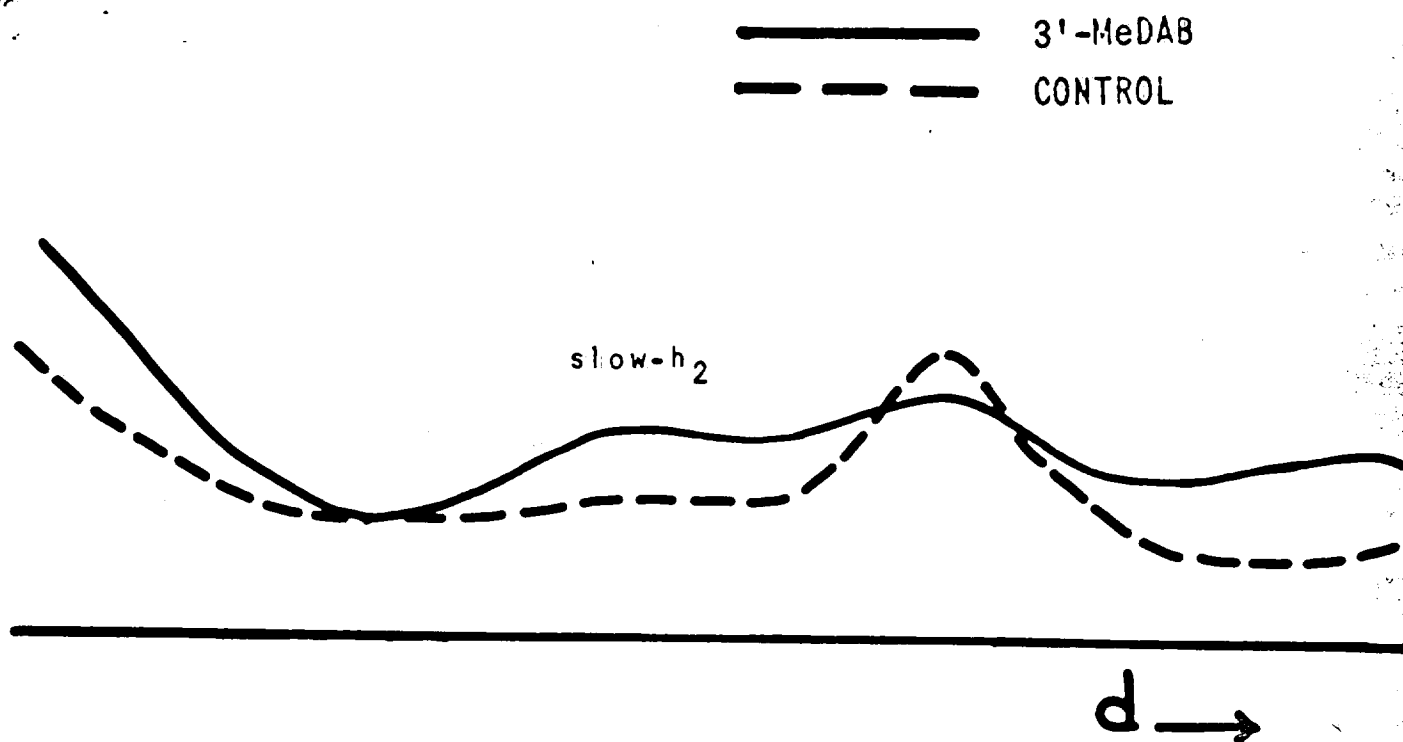
SPECTRAL EVIDENCE FOR PRESENCE OF AZO-PROTEINS AMONG SLOW-h<sub>2</sub> PROTEINS  
OF RATS FED 0.06% DAB FOR 4½ WEEKS.

(5190 mins. at 4.30 v./cm. in 0.02 μ NaV + 0.03 μ NaCl, pH 8.6)

Figure III.

1003541225





EFFECT ON SLOW-h<sub>2</sub> PROTEINS INDUCED BY FEEDING  
0.057% 3'-MeDAB FOR 13-21 DAYS

(7150 mins. at 4.30 v./cm. in 0.02% NaV + 0.03% NaCl,  
pH 8.6: 11.4% protein)

Figure IV.

1003541226

350 FIFTH AVENUE

NEW YORK 1, N. Y.

1. Budget Plan (first year) 350 FIFTH AVENUE NEW YORK 1, N. Y.  
 2. Personnel: Technician, Animal Care, Salaried  
 3. Materials: Tissue, Glassware, etc. Expensible Supplies  
 Application For Research Grant  
 Overhead 40%  
 Other

Date: March 8, 1955

1. Name of Investigator: David M. Spain, M.D. - Pathologist  
 Norman Molomut, Ph.D. - Immunologist
2. Titles and Staff Available: Experimental Animal Room; Histological Laboratory; Internal strains of mice; Experimental Histo-Pathology Laboratory; Bacteriology and Bacteriology Laboratory; Microchemistry; Tissue Culture; Microphotography.
3. Institution: Waldemar Medical Research Foundation, Inc.  
 78 Address: 16 Sintsink Drive East  
 Addition to location: Port Washington, N. Y.
4. Project or Subject: Study of Host Factors in Experimental Induction of Pulmonary Tumors in Mice.
5. Additional Requirements:

5. Detailed Plan of Procedure (Use reverse side if additional space is needed):  
 One of the basic problems in the experimental approach to the lung cancer problem is the inability with normal carcinogen to establish adequate base line controls and reproducible results as indicated by the induction of lung cancer. The techniques which have been used to date include the induction of a methylcholanthrene pellet encased in wire mesh with hooks, placed into the bronchial tree through a tracheostomy and the introduction of the methylcholanthrene by means of an impregnated thread drawn through the chest wall into the lung by a fine needle. In the former procedure, it is difficult to eliminate associated infection in the segment of the lung obstructed by the pellet. Furthermore, carcinoma as yet has not been induced with any degree of regularity by this technique. In the latter procedure, since the thread is drawn through the chest wall, subcutaneous carcinoma complicated the picture. It is proposed that we investigate a technique of direct introduction of the carcinogen in a propylene glycol or aerosol solution sprayed with slight pressure with a tracheostomy by means of a fine glass cannula directly into a bronchus. This would eliminate all of the aforementioned complications. Furthermore, with the techniques employed previously, it has been difficult to secure a significant incidence of bronchial carcinoma. It is proposed that enhancing agents such as cortisone, and/or appropriate lyophilized tumor, normal tissue or anti-sera to tumor, be given along with the carcinogen, in order to attempt to establish an adequate incidence of experimental lung cancer. Once this base line control is established, it is then proposed to repeat the procedure with those agents associated with smoking that are considered to be carcinogens. This will be done on various strains of mice and if necessary, various strains of rats.

/s/ Eleanor L. Linder  
 Business Office of the Institution

1003541227

5 continued -

If the basic technique here proposed meets with success, since in the process of smoking, areas of the lung are exposed to temporary states of relative anoxia and since individuals in their environment are exposed to carbon monoxide and other fumes which interfere with normal respiratory exchange leading to relative anoxia and since experimentally, Goldblatt and Gey have demonstrated in tissue culture that under conditions of low oxygen tension, normal cells have changed into malignant cells, it is proposed that the above experiments be repeated on animals in closed chambers with controlled oxygen tensions.

1003541228

6. Budget Plan: (first year)

Senior Biologist, Technician, Animal Care  
Animals, Feed, Bedding, MCA, glassware, etc.

Salaries

Expendable Supplies

Permanent Equipment

Overhead 10%

Other

\$ 9,250.00

1,500.00

none

1,000.00

300.00

Total, 1955 \$12,050.00

7. Anticipated Duration of Work:

Three years, P.D. - Pathologist

Norman Molomit, Ph.D. - Immunologist

8. Facilities and Staff Available:

Experimental Animal Room; cold room; Biochemical Laboratory; Inbred strains of mice; Lyophilizer; Histo-Pathology Laboratory, Immunology and Bacteriology Laboratory; Spectrophotometry; Tissue Culture; Chromatography.

9. Additional Requirements:

Staff: Pathologist, Oncologist, Immunologist, Biochemist and Pharmacologist in addition to technicians.  
John Washington, N. Y.

10. Additional Information (Including relation of work to other projects and other sources of supply):

Study of Host Factors in Experimental Induction of Pulmonary Tumors in Mice.

11. Additional Information (Including relation of work to other projects and other sources of supply):

Experimental induction of tumors in animals has been shown through our studies on inflammatory responses including immune phenomena to be definitely related to the status of host function. By means of such agents as cortisone, lyophilized tissue and tumor extracts, and anti-tumor anti-sera, tumors which ordinarily do not grow have been induced to grow and even to metastasize. The enclosed reprints of our own studies and appended bibliographies are pertinent as is the work of Baserga and Shubik (Science 121, 100) and Pomeroy (Cancer Research 14, 201). Recently, Toolan of the Sloan-Kettering Institute, following these same procedures has succeeded in growing human cancers in rats. It is believed that in order to succeed in the experimental induction of lung tumors in animals, that the technique may require some method of intervening in the normal physiologic state of the host. Indeed, this may very well be a clue to the possibility that carcinogenic substances in products such as tobacco smoke, have their influence on hosts in whom other factors of debility are existent.

With a prophylactic by means of a fine glass needle directly into a transverse would eliminate all of the aforementioned complications. Furthermore, with techniques employed previously, it has been difficult to secure a significant incidence of bronchial carcinoma. It is proposed that enhancing agents such as cortisone, and/or appropriate lyophilized tumor, normal tissue or anti-tumor, be given along with the carcinogen. If adequate incidence of experimental lung tumor is established, it is then proposed to repeat the procedure with agents associated with smoking that are considered to be carcinogenic. This will be done with various strains of mice and if necessary, various sources of agents.

Signature: /s/ Norman Molomit

Director of Project

/s/ Florence Lazere

Business Officer of the Institution

Reprints Enclosed

1. "Cortisone Effect on Pneumonitis Produced in Mice by Exposure to a High Oxygen Atmosphere", Warshaw, L. J., Molomut, N., and Spain, D. M., Proc. Soc. Exp. Biol. Med., 80, 341 (1952).
2. "Effect of Previously Injected Immune Serum and Tissue on the Survival of Tumor Grafts in Mice", Kaliss, N., Molomut, N., Harriss, J. L. and Gault, S. D., J. Nat. Cancer Inst., 13, 847 (1953).
3. "Induction of Metastases from Sarcoma I in C37 BL/6 Mice", Molomut, N., Spain, D. M., Gault, S. D. and Kreisler, L., Am. J. Pathology, 30, 375-389 (1954).
4. "Preliminary Report on the Experimental Induction of Metastases from a Heterologous Cancer Graft in Mice", Molomut, N., Spain, D. M., Gault, S. D. and Kreisler, L., Proc. Nat. Acad. Sciences, 38, 991 (1952).
- 5 "Some Basic Biologic Effects of Cortisone as Related to Pulmonary Disease" Spain, David M., Diseases of the Chest, 23, 270 (1953).

1003541230

TOBACCO INDUSTRY RESEARCH COMMITTEE

150 EAST FORTY SECOND STREET NEW YORK 17, N. Y.

Salaries  
Expendable Supplies  
Application For Research Grant  
Permanent Equipment  
Other

\$13,200.00

\$10.00

\$22.00

\$,482.50

# 124(a)

Total \$13,832.50

Date: May 15, 1956

7. Anticipated Duration of Work:

1. Name of Investigator:

Dr. Herbert J. Spoor (1)

Dr. Alexander Borota (2)

Dr. Thomas H. McGavack (3)

8. Investigator's Present Address:

2. Title:

(1) Assistant Professor of Dermatology; (2) Associate Attending Dermatologist;  
(3) Director, New York Medical College, Metropolitan Medical Center  
Research Unit.

3. Institution

& Address:

New York Medical College  
Metropolitan Medical Center Research Unit  
at Bird S. Coler Hospital  
Welfare Island 17, New York

4. Project or Subject:

9. Detailed Requirements:

This study will deal with the following questions: (1) How does smoking affect the oral mucosa of aged patients; (2) Can a possible correlation be made between smoking and the development of cancerous or pre-cancerous lesions in the oral mucosa; (3) What is the frequency or association of smoking and the development of pre-cancerous or cancerous lesions of the oral mucosa?

10. Additional Information (Including relation of work to other projects and other sources of support):

5. Detailed Plan of Procedure (Use reverse side if additional space is needed):

No other source of money.

See attached protocol.

Signature

Dr. Herbert J. Spoor  
Dr. Alexander Borota  
Dr. Thomas H. McGavack

Approved by the Institution

1003541231

# INFLUENCE OF SMOKING ON THE ORAL MUCOSAE OF AGED PEOPLE

## FOREWORD

Smoking or the use of tobacco in any form as a cancerogenic factor has been studied by a number of investigators. The results of these studies have been widely variant in their conclusions. Some workers have considered the use of tobacco an important causative agent in the development of cancer of the lung and mucous membranes of the oral cavity. Other workers have felt that it was a relatively unimportant cancerogenic agent. These contributions expressing somewhat opposite viewpoints all arise from good scientific sources. Therefore, it would seem apparent, even to the casual observer, that in the studies which have been carried out, factors other than tobacco may have been completely overlooked or misinterpreted. In view of the confusion in the field, it seems wise to direct a study towards answering the following questions: (1) How does smoking affect the oral mucosa of geriatric patients; (2) Does smoking alone produce cancer or precancer of the oral mucosa; (3) Does smoking alone predispose to cancer or precancer formation of the oral mucosa and (4) What is the role and importance of other cancerogenic factors in the domestic and occupational environment in the development of oral cancer?

## DESIGN OF THE PRESENT INVESTIGATION

In the present study it is planned to examine by methods later detailed the oral cavities of a large number of people within a geriatric population. A certain percentage of these people have never smoked and the others have been smokers or users of tobacco in some form to a greater or a lesser extent. All subjects will be chosen from the wards of the Bird S. Color Hospital. There are several obvious reasons why the oral cavity has been chosen for this type of observation:

1. The oral cavity is exposed to the highest concentration of smoke and other inhaled material;
2. It may react to irritation from a wide variety of causes with no discernible response; or an inflammatory reaction with cancer or pre-cancer formation;
3. It is an area which is easy to examine and reexamine accurately and continuously. Moreover, this is an area from which objective biopsy studies may be made with relative ease. These subjects will be divided into groups in relation to their use of tobacco. All of those who do not smoke at all will be placed in a single group. The remainder will be divided into sub-groups predicated upon their smoking habits.

In each subject historical data will be gained regarding (a) race, age and sex; (b) occupational and domestic environmental history; (c) duration and intensity (amount of the use of tobacco in any form and (d) familial history, which is to include both smoking and non-smoking members in so far as obtainable.

Physical examination will include a study of (a) body habitus, natural pigmentation, eye color, personal distinguishing characteristics; (b) careful examination of the oral cavity and readily visible adjacent structures; (c) a general examination to detect other lesions of skin or mucous membranes and (d) general medical and dermatological examinations.

1003541232

Influence of Smoking ..... Cont'd.

Laboratory examinations will be employed when and as necessary to establish a diagnosis. These will include:

1. Routine chemical and serological examinations.
2. Where indicated, special tests will be done, such as cultures of the mouth and of the lesions themselves.
3. Biopsies of those lesions which clinically demand removal; biopsies of lesions which are suspected of being cancerous or pre-cancerous.

Data collected as above will be analyzed to answer questions which have been raised in connection with the use of tobacco.

At least 1,500 patients will be studied so that deductions can probably be made statistically valid.

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## TOBACCO INDUSTRY DATA

## 6. Budget Plan:

130 EAST FORTY SECOND STREET

Salaries	\$15,200.00
Expendable Supplies	850.00
Permanent Equipment	500.00
Overhead	2,482.50
Other	15%
Total	\$ 19,032.50

Date: May 14, 1976

## 7. Anticipated Duration of Work:

At least one year.

## 8. Facilities and Staff Available:

All available at Research Unit at Bird S. Color Hospital

- (1) and on the wards of the same hospital. (2) (Luncheon) ophthalmologist;  
 (3) Director. See also attached application for research, this Medical Center

## 9. Additional Requirements:

None

This study will deal with the following questions: (1) How does smoking affect the oral cavity of oral cancer? (2) The epidemiologic correlation of oral cancer with the development of a program of pre-operative diagnosis in the oral cavity? (3) What is the frequency of association of smoking and the development of pre-operative or surgical lesions of the oral cavity?

## 10. Additional Information (Including relation of work to other projects and other sources of supply):

See attached Plan of Procedure (Use reverse side if additional space is needed):

No other sources of supply.

See attached protocol.

/s/ H. J. Spoor  
 Signature /s/ Alexander Borota  
 Director of Project /s/ Thomas H. McGavack

Business Office of the Institution

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CROSS REFERENCE SHEET

Name or Subject

Leslie H. Squires

Regarding

Res. Grant

SEE

Robt. C. Wilson

1003541235

Application For Research Grant

Overhead

City (New York), Date: September 30, 1955

Date: September 30, 1955

1. Name of Investigator: William E. Smith, M.D.

2. Anticipated Duration of Work

2. Title:

3. Institution

Facilities, maintenance of records and statistical services will be provided by the University of Pennsylvania, Philadelphia, Pa. 19104  
& Address: 170 Glenwood Road, Englewood, New Jersey

4. Project or Subject: Experiments pertaining to lung tumors.

1. Exploration of specificity of a rapid test for carcinogenicity of chemicals.

2. Search for a virus in pulmonary adenomas of mice.

3. Investigation of dietary deficiency of choline in relation to lung tumors.

5. Detailed Plan of Procedure (Use reverse side if additional space is needed).

10. Additional Information (Including provision of work to other projects and other sources of support).

1. Enumeration of sebaceous glands and observations upon hair loss within ten days after application of test materials to mouse skin have provided a rapid means for predicting the relative carcinogenic potencies of derivatives of petroleum, coal tar and cigarette smoke, as indicated in attached material (Smith, W.E.; Sebaceous gland activity as a measure of the relative carcinogenicity of certain oils and tars. Bull. N.Y. Acad. Med., in press).

A purpose of the present project is to explore the specificity of these early changes through study of pure chemicals of varying carcinogenicity and structurally related but non-carcinogenic compounds. It is also intended to apply this technique for further study of derivatives of cigarette smoke. As the originator of this technique, I have been offered an appointment as a visiting professor in the University of Paris, to work in the laboratory of Professor R. Truphot at the Faculté de Pharmacie, where studies of the potential carcinogenicity of cigarette smoke are being conducted under the auspices of the Institut National d'Hygiène de France. Supplies of pure chemicals of varying carcinogenicity have been offered by Professor A. Lacassagne, Director of the Institut du Radium, in Paris, which possesses a unique collection of such compounds.

2. Use of the tissue transplant technique for ascertaining carcinogenicity of materials upon lung tissue is commented upon in attached articles (Smith, W.E.; The tissue transplant technique as a means of testing materials for carcinogenic action, Cancer Research 1949, 9, 712-723; Lung cancer with special reference to experimental aspects, Arch. Indust. Hyg. & Occup. Med. 1952, 5, 209-217; Business Officer of the Institution

1003541236

Evaluation of claims for occupational factors in cancer of the lung, Acta Union Internat. contre le Cancer, 1953, 9, 476-484).

Cancers of the nasal sinuses and lung have appeared to be an occupational risk among employees of a nickel refinery. By means of the tissue transplant technique, I have in unpublished experiments induced transplantable adenocarcinomas by intramuscular implantation of nickel powder together with fetal lung tissue from C strain mice. I now am carrying one of these tumors in the third generation of new hosts. I wish to learn whether this tumor carries a virus capable of inducing similar growths. I propose to test for the presence of such an agent by exposing pulmonary tissue from mouse embryos of the A strain to be extracts of this tumor that was induced from pulmonary tissue from the C strain. The exposed pulmonary tissue will be transplanted into A strain hosts in an endeavor to avoid tumors from the growth of any intact C strain cells that may inadvertently be present in the extracts.

3. Clinical and experimental studies have indicated that several types of tumors may result from dietary deficiencies. Primary carcinomas of the lungs have been reported in 38% of rats fed a diet deficient in choline (Copeland and Salmon, Amer. Journ. Path. 1946, 22, 1059-1079). No lung tumors were found in control animals fed a diet supplemented with choline.

If these claims can be corroborated, they would open an interesting field of experimentation upon etiology and possibly treatment of lung tumors. It is therefore intended:

- a) to repeat the feeding experiment of Copeland and Salmon, using a group of 100 rats (50 fed a diet deficient in choline, 50 fed the same diet but supplemented with choline).
- b) to conduct a similar experiment with 100 mice of the A strain with special reference to any alteration in the frequency of pulmonary adenomas among them.

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## 6. Budget Plan:

TOBACCO INDUSTRY  
350 FIFTH AVENUE

Principal investigator	\$10,000
Histologist-technician	3,000
Animal caretaker	500 part salary
Salaries	
Expendable Supplies (animals, chemicals)	1,200
Permanent Equipment	500
Overhead (10%)	1,520
Other (travel, investigator & family, one way)	1,200
Total	17,920
per year	

1. Name of Investigator: William E. Smith, M.D.  
7. Anticipated Duration of Work:

Two years beginning March 1, 1956

## 8. Facilities and Staff Available:

3. Laboratory facilities, maintenance of animals and histological services will be provided by the Faculte' de Pharmacie, Universite' de Paris, Av. de l'Observatoire, Paris 6<sup>e</sup>, France.

4. Nature of Subject: Experiments pertaining to lung cancer.

## 9. Additional Requirements: specificity of a rapid test for carcinogenicity of chemicals.

None

5. Investigation of dietary deficiency of choline in relation to lung cancer.

6. Detailed Plan of Procedure (Use reverse side if additional space is needed).

## 10. Additional Information (Including relation of work to other projects and other sources of supply):

The Institut National d'Hygiene de France has appointed Professors Lacassagne, Oberling and Truhaut to conduct, in their respective laboratories, studies upon the potential carcinogenicity of smoke from various types of tobacco and to identify any carcinogens that may be present. Professor Truhaut has invited me to work in his laboratory in order to apply my rapid test for carcinogens to some of the materials developed in the course of this program.

Extensive studies on molecular structure in relation to carcinogenicity have been conducted for many years at the Institut du Radium in Paris. As a result, this institution possesses a unique collection of carcinogens and related compounds. The director, Professor Lacassagne, and his associate, Professor Buu Hoi, have consented to supply appropriate samples and advice for intelligent selection of compounds to explore the specificity of the rapid test for carcinogenicity described in this application. The Faculte' de Pharmacie, where studies of the potential carcinogenicity of cigarette smoke are being conducted under the auspices of the Institut du Radium, in Paris, which possesses a unique collection of such compounds. The applicant speaks French. Supplies of pure chemicals of varying carcinogenicity have been offered by Professor A. Lacassagne, Director of the Institut du Radium, in Paris, which possesses a unique collection of such compounds.

Signature: s/ William E. Smith, M.D.

Use of the mouse transplant technique for projecting carcinogenicity of chemicals upon lung tissue is presented. The mouse transplant technique as a means of testing chemicals for carcinogenicity. Cancer Research 1949, 9, 722-723; lung cancer with special reference to experimental aspects, Arch. Indust. Hyg. & Gen. 1947, 1, 200-217.

Business Officer of the Institution

1003541238

C O P Y

NEW YORK UNIVERSITY-BELLEVUE MEDICAL CENTER  
NEW YORK UNIVERSITY POST-GRADUATE MEDICAL SCHOOL  
INSTITUTE OF INDUSTRIAL MEDICINE  
550 First Avenue, New York 16, N. Y.  
ORegon 9-3200.

September 30, 1955

Dr. Robert C. Hockett  
Tobacco Industry Research Committee  
5320 Empire State Building  
New York 1, New York

Dear Dr. Hockett:

Thank you for your advice in our recent telephone conversation. I am submitting herewith an application for a research grant. The background for this proposal is as follows.

Last fall, Professor Lacassagne visited me to discuss my rapid test for estimation of carcinogenic activity of chemicals. His interest stems from the possibility of applying this procedure in studies of the potential carcinogenic properties of cigarette smoke now being conducted in his and related laboratories in the University of Paris. Chemical fractionation of cigarette smoke is being conducted there by Professor Buu Hoi, one of the outstanding chemists in the world in the field of carcinogens, who has had many years experience in the preparation of carcinogens and their analogs for the studies on relation of molecular structure to carcinogenic activity which have long been a major program in Professor Lacassagne's Institute.

Last May I visited Professors Lacassagne and Buu Hoi to inquire into the possibility of obtaining for rapid test fractions of cigarette smoke prepared by them and, more particularly, pure chemicals of varying carcinogenicity from their extensive collection in order to evaluate specificity of the rapid test.

These associations have been most cordial and stimulating, and Professor Lacassagne has assured me of laboratory facilities if I wished to do the work in Paris. He offered to supply appropriate materials, and advised that the best space would probably be available in Professor Truhaut's laboratory. Professor Truhaut conducts studies of industrial toxicology for the French government and is one of three men appointed by the government to investigate biological properties of tobacco smoke.

Recently, I received the attached letters from Professor Truhaut offering me facilities in his laboratory, provided that I can obtain a grant to cover my salary and the purchase cost of animals. He offers to cover the cost of maintenance of animals and preparation of histological sections.

In drawing up the proposed budget in the attached application, I have, however, introduced items for supplies and technicians' salaries.

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I believe this is wise to assure prompt supply and service. Since this application requests more support than Professor Truhaut stated necessary, I feel confident that it will receive the signature of the proper officer of the University of Paris. I am forwarding the application to you at this time in the hope that it may be considered at the forthcoming meeting of your committee. In the event that favorable action might be taken, I would of course recognize that finalization must await return of a properly executed copy from the University of Paris.

Sincerely yours,

s/. William E. Smith, M.D.

Enclosures.

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TIRC Project #114

William E. Smith, M.D.

Biography

**REDACTED**

1934 A. B., Princeton  
1938 M.D., Johns Hopkins  
1940-43 Research fellow in bacteriology, Harvard Medical  
School  
1943-47 Assistant in pathology, Rockefeller Institute for  
Medical Research  
1947-49 Associate, Sloan-Kettering Institute for Cancer  
Research  
1949-53 Assistant professor of industrial medicine, New York  
University  
1953-55 Associate professor of industrial medicine, New  
York University

Publications

List attached. (on file with R. C. H.)

References

Dr. Peyton Rous  
Rockefeller Institute for Medical Research  
66th Street and York Avenue  
New York, New York

Dr. George T. Pack

**REDACTED**

Dr. Harold L. Stewart  
National Cancer Institute  
Bethesda, Maryland

1003541241



TOBACCO INDUSTRY RESEARCH COMMITTEE  
150 East Forty Second Street  
New York 17, N. Y.

Application for Research Grant

Date: July 29, 1959

1. Name of Investigator: Douglas H. Sprunt, M. D.
2. Title: Professor of Pathology
3. Institution & Address: University of Tennessee  
64 South Dunlap, Memphis, Tennessee
4. Project or Subject: Multiple Repeated Pulmonary Irritation as a Cause of Lung Cancer.
5. Detailed Plan of Procedure: This study concerns the effect upon the mammalian lung of chronic irritants, instilled intratracheally over a period of months. It is in some respects a follow-up on the statement of Winternitz and others ("The Pathology of Influenza," Yale University Press, 1920) following the influenza epidemic of World War I, that the metaplasia and proliferation that occurred in the small bronchi might well result in bronchogenic carcinoma.

We have been able to produce atypical, metaplastic and proliferative changes in the lungs by the injections of bacterial toxins (staphylococcal, streptococcal and diphtherial), vaccinia virus and 0.1 normal hydrochloric acid into the tracheae of rabbits. The amount injected is 1 cc., and intratracheal instillation is made with a syringe and needle under direct vision. Preliminary studies with India ink and trypan blue dyes have demonstrated that this method, aided by the normal inspiratory movements of the animals spreads injected fluids into large portions of both lungs.

After an irritant is injected, prophylactic antibiotics are given to protect against secondary infection. The rabbits are re-operated at two-month intervals until a total of five injections has been given. Then the animals are allowed to live until natural death intervenes, when gross and microscopic study of lungs and internal organs is made.

Since the essence of this experiment concerns the effect of long-term, chronic irritation, the number of animals available for comment at present is quite small. To date, however, many instances of abnormal changes in cells, metaplasia and proliferative changes have been observed (see illustrations). An encouraging factor is the high incidence of metaplastic proliferations produced in young animals after only short-term irritation, often within ten days after instillation of the irritant.

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In the evaluation of these lesions, the importance of cytologic and morphologic standards is important. In situ or carcinoma of the lung has much the same diagnostic criteria as that of the cervix uteri. In this department, we have had an opportunity to study, in connection with the Memphis and Shelby County Cancer Survey, a large number of biopsies from women with various changes in the cervical epithelium. We have examined smears on over 150,000 women and have examined biopsies of the cervix in over 2500 of these women. On the basis of our criteria for the diagnosis of carcinoma-in-situ of the cervix, we do not believe any of the lesions that have so far occurred in the rabbits should be called carcinoma-in-situ, but the epithelia as shown in the accompanying illustrations are markedly altered and are not merely regenerative. It is our belief that some of these changes would have progressed to cancer, had the animals lived.

Since these lesions have been produced by chronic irritation, our results lead us to disagree with Spain, (Am. Rev. Tuberc. 79:591, 1959) who recently advanced the concept that the bronchial epithelium follows separate lines of response to injurious stimuli, the first being that of simple regeneration, the second being that of malignant neoplasia. This represents a new approach, since much of the thinking prior to Spain's article had been directed toward that of regenerative proliferation progressing into neoplasia, with chronic disease producing multiple injuries, then regeneration, metaplasia, atypism and finally, frank malignancy occurring in consequence.

In apposition to this, in a series of 50 cases of lung cancer, Spain found many areas of atypism and carcinoma-in-situ situated in the pulmonary tissues independent of the primary cancer, but in a parallel series of cases of chronic lung diseases, only regenerative changes were found. Thereupon, postulation was made that a specific carcinogen produced many pre-malignant areas in the lung cancer cases besides the primary carcinoma, and that conversely, chronic lung disease per se does not lead to cancer, but elicits a separate epithelial response leading only to regenerative proliferative changes. However, in both series, significant regenerative changes in his series of lung cancer cases by the suggestion that perhaps cigarette smoke contains both non-specific (chronic) irritants as well as a specific carcinogen.

The article of Berkheiser (Cancer, 12; 1959) discusses the more generally accepted concept that injury to the bronchiolar and pulmonary epithelium leads to abnormal changes in the epithelium which resemble cancer. It is our belief, however, that although as pointed out by Andrial (Arch. Path. 86: 94; 1959) that while the metaplasia produced by influenza disappears shortly after the infection is cured, if the pulmonary epithelium were repeatedly irritated, as we are doing, that the lesions would progress to cancer.

6. Budget Plan:

Salaries	\$13,600.00
Expendable Supplies	2,300.00
Permanent Equipment	3,000.00
Overhead at 15%	2,895.00
Other	400.00
Total	\$22,195.00

Four additional years for a total of: \$19,320.00 each year

7. Anticipated Duration of Work: Five years: January 1, 1960 to December 31, 1965.

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8. Facilities and Staff Available: We have a well-equipped, air-conditioned animal room and laboratory in use in this work now. There is adequate space for expanding this program. The program has been supported by a grant from the United States Public Health Service (\$7000.00) since March 1, 1957. This grant expires February 2, 1960.

The graduate student in this program, Mr. James M. Parsons, has been working with this program since its inception, three years ago. He is taking a joint graduate and medical program, spread over nineteen academic quarters, which will lead to both an M.D. and Ph.D. degree.

9. Additional Requirements:
10. Additional Information (Including relation of work to other projects and other sources of supply):

The graduate student, Mr. James Parsons, who has been working on this project since its beginning, is now a little over halfway through his academic program. The program is expected to run five years. After the first year, the budget would be reduced from \$22,195.00 to \$19,300.00, as we would not need the \$3000.00 for permanent equipment, but we have allowed \$500.00 for eventualities. The \$3000.00 is to be used to purchase 70 additional rabbit cages. We have 101 in use now, but this number should be increased. We have adequate air-conditioned quarters for this addition.

If this grant is made, we would not expect to seek further aid for this project from the United States Public Health Service. We are enclosing a book of photographs from the lesions found in rabbits in this experiment. We do not believe any of these are lung cancer or even carcinoma in situ, but they do show a number of abnormal changes.

The amount of money in salaries is as follows:

Graduate Student	\$3600.00
Technician	4000.00
Animal Dieners (2)	3400.00
Maid	1800.00
Retirement	800.00
6 1/4%	
Total	\$13,600.00

Consumable supplies comprise glassware, antibiotics and animal food.

The \$400.00 for "Other" is for travel of the graduate student to medical and scientific meetings.

/s./ Douglas H. Sprunt  
Director of Project  
Douglas H. Sprunt, M.D.

/s./ Cecil Q. Tipton  
Business Officer of the Institution  
Cecil C. Tipton, Administrative Assistant

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\*Salary to be used for additional help and not for the investigator. Date: January 11, 1955

7. Anticipated Duration of Work: \_\_\_\_\_

1. Name of Investigator: **Dr. J. Manly Stallworth, M.D.**

8. Facilities and Staff Available: \_\_\_\_\_

2. Title: **The Effects of Cigarette Smoke on the Peripheral Vascular System, funds.**

3. Institution: **Cardiovascular Dept., Medical College of South Carolina**  
& Address: **16 Lucas Street, Charleston, South Carolina**

4. Project or Subject: **A comparison of the effects of smoking cigarettes with those of subcutaneous injections of adrenalin chloride on the peripheral vascular system of normal males in an effort to determine what the effects of smoking a cigarette is equivalent to in terms of a known vasoconstricting agent.**

10. Additional Information (including relation of work to other projects and other matters of record): \_\_\_\_\_

5. Detailed Plan of Procedure (Use reverse side if additional space is needed): **Comparison is to be made of the effects of smoking two standard, popular brand, non-filtered, cigarettes at a natural rate, and a standard dose (amount to be determined) of sub-cutaneous adrenalin chloride on the peripheral vascular system of human subjects. Subjects will be normal male medical students or members of our hospital house staff, the majority of whom will be habitual smokers but will include non-smokers for comparison. Utilized for this comparison will be pulse rate, blood pressure, skin temperature, and plethysmographic determination of pulse volume. The skin temperature and the pulse volume are to be obtained from the digits of both the upper and lower extremities. Serial determinations are to be made on cigarette fasting subjects before, during and after both the smoking and adrenalin administration, with a minimum of twenty-four hours separating the two.**

Signature \_\_\_\_\_  
Director of Project \_\_\_\_\_

J. Marly Stallworth, M.D.

**Business Officer of the**

H. M. Pirloy

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6. Budget Plan:

Salaries	\$3,600.00*
Expendable Supplies	200.00
Permanent Equipment	0.00
Overhead (8% total grant)	304.00
Other	0.00
Total	\$4,104.00

\*Salary to be used for additional help and not for the investigator.

7. Anticipated Duration of Work:

Name of **Twelve Months.** **Manly Stallworth, M.D.**

8. Facilities and Staff Available:

7. Title: **Direct writer plethysmograph machine, thermocouple and other permanent equipment are on hand or on order from other grant funds. Two medical fellows in Cardiology - one full time, one part time - available to run the comparative studies.**

3. Institution

& Address

4. Project or Subject

9. Additional Requirements:

**None.**

10. Additional Information (Including relation of work to other projects and other sources of supply):

5. Detailed **Current investigation by colleagues under another grant is under way in an effort to establish a method of measuring vessel constriction utilizing microphotography of conjunctival vessels. When this is proven and available further opportunity will be afforded to study the effect of smoking on peripheral vessels and comparison of smoking and vasoconstrictor drugs.**

Signature /s/ J. Manly Stallworth  
Director of Project

**J. Manly Stallworth, M.D.**

Business Officer of the Institution

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**CONFIDENTIAL**

TIRC Grant # 20R1

Progress Report #2  
August 3, 1956

Dr. Fredrick J. Stare

Harvard School of Public Health

"Progress Report on Research Grant-in-Aid entitled 'Experimental Studies on Cancer utilizing a new technique to see if various tars extracted from tobacco may incite the formation of lung tumors.'"

*Objectives?*

- 1) Additional emulsions of tobacco tar concentrate have been made using different emulsifying techniques and with and without carrier oils. None of these were suitable in animals, presumably because of the nicotine content. Although some animals survived, all of these experiments were terminated because insufficient numbers would have seriously weakened the statistical significance of the results.
- 2) The neutral fraction of the tobacco tar concentrate was prepared as follows: the toluene was removed in vacuo and the tars were vigorously agitated with cyclohexane. The cyclohexane solution was extracted three times with 2N HCL, three times with 2N NaOH, and finally with water. The cyclohexane was removed and the residue was used in subsequent studies. Although emulsions could be made in a manner similar to that used in earlier work, it was felt that it would be desirable to devise a procedure which would circumvent prolonged elevated temperatures. The following method was adopted: the proper amount of the neutral fraction was added to 7 ml. of 100 percent of ethylalcohol which contained 2 to 4 gms. of Pluronic - F68 (a polyoxyethylene - polyoxypropylene glycol). The mixture was warmed to effect solution and then was rapidly injected from a sterile syringe into 95 ml. of sterile 5 percent dextrose. This results in a colloidal dispersion of the neutral fraction which is well tolerated by animals when given intravenously. Several strains of mice have been given 9 intravenous injections of this preparation and are now under observation. Control animals which have received all emulsion ingredients except the tar fraction are also being observed. The alcohol in the emulsion can be removed easily by the use of a rotating low temperature evaporator; however, no adverse effect of the alcohol has been observed.
- 3) A high speed stirrer - homogenizer has been constructed for use in preparing the emulsions which contain carrier oil or the original tar concentrate. Work is still underway to develop a still more efficient stirrer-impact device, for the tars remain troublesome because of viscosity characteristics. We still feel it would be desirable to give some of the animals tars which have been purified as little as possible.
- 4) From the work which has been concluded with a variety of known carcinogens given intravenously (not prepared from tobacco tars), it is apparent that even at elevated doses some do not give rise to an appreciable tumor incidence. Furthermore, one cannot predict which agents will be effective. Therefore, it is obvious that negative results with the

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tobacco tars will certainly not prove the absence of carcinogens. However, to date, no tumors have been found in any of the animals injected with tobacco tar extract. We intend to continue the animal experiments using both the "colloidal" and emulsion preparations of the neutral fraction until the expiration of this grant.

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Industry research laboratory because we do not have sufficient space or personnel to extract these tars in our own laboratories. To date we have made no attempt to obtain tobacco tars from any tobacco research laboratory but are certain that this could readily be done and we would welcome the help of the Tobacco Industry Research Committee in this connection.

Our new experimental technique would be used as follows: Various tobacco tars would be dissolved in various oils, emulsions made, and the tar containing emulsions given by vein to adult male rats. The dosage and frequency of injections would be governed by our previous experience in this technique using other carcinogens. After four months, representative animals will be autopsied and examined for tumors, and this procedure will be repeated monthly or as often as thought necessary.

Address: 605 Huntington Avenue, Boston 12, Massachusetts  
Department of Nutrition, Harvard School of Public Health

Project or subject: Experimental studies on cancer utilizing a new technique to see if various tars extracted from tobacco may incite the formation of lung tumors.

2. Detailed Plan of Procedure (Use reverse side if additional space is needed)

This laboratory in recent years has developed a new technique for the experimental production of cancer. So far our studies have been limited to cancer of the breast. One of the unusual features of this technique is that with cancer of the breast the tumor can be produced in 100% of the animals and in ~~known~~ the remarkably short time of 16 weeks. The technique stems from our long and pioneering research in the preparation of emulsions of fat that can be safely injected directly into the blood stream. The main purpose of these studies has been to develop a concentrated source of calories for use in patients unable to consume adequate amounts of food.

But in addition to supplying calories, fat in a suitable emulsion might be used as a vehicle for fat soluble chemicals or drugs that one might wish to give directly into the blood. This is where cancer came into our research program.

DMBA (dimethyl benz anthracene) is a known carcinogen. It is fat soluble as are most known chemical carcinogens. When it is dissolved in fat along with some estrogen, the material finely emulsified and given to rats by vein, adenocarcinomas of the breast result in 100% of the animals within 16 weeks (See attached reprint).

We propose to utilize this new technique to see if various tars extracted from tobacco will give rise to cancer of the lung or cancer in any other body tissue. These researches would have to be cooperative with some tobacco

## 6. Budget Plan:

## Annual

Salaries	
Expendable Supplies	\$6,000
Permanent Equipment	4,000
Overhead	500
Other	* 25%
Pension and Social Security	390
Total	\$13,613

## 7. Anticipated Duration of Work:

Preliminary results might be available in a year's time but the necessity of working out adequate dosages might require longer and hence this exploratory study should be planned for a minimum of two years.

## 8. Facilities and Staff Available:

The Department of Nutrition has seven well equipped laboratories and is active in a great variety of biological researches including cancer. Staff, technical assistants, and research students number 65-70 individuals per year. The senior staff includes besides the two investigators listed on this application, Drs. J. Mayer, D. M. Hegsted, G. V. Mann, S. B. Andrus, S. Gershoff, and M. Trulsson. All of the staff are available for consultation.

It might be mentioned that the department has its own full-time pathologist (Dr. Andrus) and laboratory for pathology.

## 9. Additional Requirements:

None at this time--if the results look promising additional research can be planned.

## 10. Additional Information (Including relation of work to other projects and other sources of supply)

This research would bear some relation to current researches in this laboratory on cancer of the breast.

If these researches should turn out to be negative, in our opinion, they might constitute experimental evidence that tobacco tars have little direct relation to cancer of the lung. If the results should be positive it should be possible to fractionate the tars and see what components are involved. Procedures might then be designed for the removal of the offending substance if one is found.

No other funds have been requested for support of this project.

Signature

(s) Frederick J. Stare

(s) John C. Snyder

Assistant Dean

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TOBACCO INDUSTRY RESEARCH COMMITTEE  
350 FIFTH AVENUE NEW YORK 1, N. Y.

6. Budget Plan:

Renewal

Salaries & Stipends  
Application For Research Grant

#20-R1-50

4,120

1,100

1,000

1,000

1,000

Date: **October 10, 1955**

for homogeneous mix.

1. Name of Investigator: **Frederick J. Stare, Ph.D., M.D., and Robert Pl Ceyer, Ph.D.**

7. Anticipated duration of work.

This is really the second year of exploratory research. At the end of this year, we  
ex2: Title: **Professor of Nutrition and Assistant Professor of Nutrition, respectively.**

8. Facilities and Staff Available:

Facilities: The Department of Nutrition and Harvard School of Public Health and

is3. Institution: **Department of Nutrition, Harvard School of Public Health**

& Address: **1 Shattuck Street, Boston 15, Massachusetts**

Staff: Staff, technical assistants, and research assistants. Some individuals  
per year. The regular staff includes, besides the two listed in this  
application, Dr. J. Mayer, D. M. Fogarty, E. A. Matarazzo, and Dr. S. S.

ex4: Project or Subject: **Continuation of experimental studies of the possible carcinogenic  
action of cigarette smoke condensate using emulsions of this material intravenously  
in rats and mice. Pathology.**

9. Additional Requirements:

none

5. Detailed Plan of Procedure (Use reverse side if additional space is needed):

10. Additional Information Regarding Tobacco Use: The tobacco used for the purpose of this study  
Cigarette smoke condensate obtained from the Ecusta Paper Corporation will be  
rendered free of toluene by means of a vacuum system employing liquid nitrogen. Some  
of this material will be used in an emulsified form intravenously in rats and mice.  
Comparable animals will receive tar-free emulsions which will contain an amount of  
nicotine comparable to that found in the tar emulsion. If possible, the use of  
graded doses of tar or nicotine to build up tolerance in the animals will be avoided.  
Two slow injection machines are now available in case it becomes necessary to in-  
fuse the animals over a longer period of time than can be obtained with a hand  
syringe. One of the attending researchers will be assigned to

Some of the toluene-free condensate will be rendered nicotine-free, if possible.  
Although any procedure used for this purpose might change the composition of the  
tobacco tars somewhat, it would appear worthwhile if nicotine toxicity proves a  
continued problem. Some discussion of this problem was carried on recently with  
members of the Ecusta Paper Corporation.

The animals used will be Sprague-Dawley male and female albino rats, and C<sub>3</sub>H and  
DBA-1 mice. Additional strains of mice will be used as warranted. The animals will  
be maintained on stock ration except for a few groups which will be fed synthetic  
ration. Constant examinations for tumors will be made and autopsies will be done  
on the animals.

Some time will be expended to improve existing emulsification equipment. This is  
thought worthwhile because of the physical characteristics of the tars which make  
emulsification difficult

Business Office of the Institution

1003541252

# 6. Budget Plan:

TOBACCO TARS BY EXTRACTION  
350 FIFTH AVENUE

Salaries & Pensions

\$6,390

Expendable Supplies

4,000

Permanent Equipment

500

Overhead 25%

2,973

Other Development equipment

1,000

for homogenizing tars.

Total

14,863

## 1. Name of Investigator

## 7. Anticipated Duration of Work:

This is really the second year of exploratory research. At the end of this year, we expect to know if this technique is worth pursuing on this problem.

## 8. Facilities and Staff Available:

Facilities: The Department of Nutrition has seven well-equipped laboratories and is active in a great variety of biological researches, including cancer.

Staff: Staff, technical assistants, and research students number 65-70 individuals per year. The senior staff includes, besides the two investigators listed on this application, Drs. J. Mayer, D. M. Hegsted, T. B. VanItallie, S. B. Andrus, S. Gershoff, and M. Trulson. All of the staff are available for consultation. It might be mentioned that the department has its own full-time pathologist (Dr. Andrus) and laboratory for pathology.

## 9. Additional Requirements:

none

## 10. Additional Information (Including relation of work to other projects and other sources of supply):

This research is related to current researches in this laboratory on cancer of the breast. The animals will be given tar-free cigarettes which will be given in amount of 10 mg. per day. If these researches should turn out to be negative, in our opinion, they might constitute experimental evidence that tobacco tars have little direct relation to cancer of the lung. If the results should be positive it should be possible to fractionate the tars and see what components are involved. Procedures might then be designed for the removal of the offending substance if one is found.

No other funds have been requested for support of this project. Although any procedure for the isolation of the tobacco tars component, it is not certain if nicotine toxicity poses a continued problem. Some fractionation of this problem was carried out recently with members of the Fruton group.

The animals used will be 100g. body weight male and female white rats, 400g. and 400g. mice. Additional quantities of mice will be used as warranted. The animals will be maintained on stock diet and water for a few groups which will be fed synthetic diet. Constant observation of the animals will be done by Frederick J. Stare.

Signature

Director of Project

Some time will be expended to fractionate existing purified equipment. This is thought worthwhile because of the chemical characteristics of the tars which make purification difficult.

Business Officer of the Institution

Committee:

Dr. Kotin  
Dr. Jacobson  
Dr. Lynch

TOBACCO INDUSTRY RESEARCH COMMITTEE

150 East Forty-Second Street

#274

New York 17, N.Y.

Application for Research Grant

Date: April 30, 1960

1. Name of Investigator: Arthur A. Stein, M.D.
2. Title: Professor of Pathology
3. Institution & Address: Albany Medical College  
New Scotland Ave.  
Albany, New York
4. Project or Subject: The induction of bronchial epithelial hyperplasia  
by human cancerous bronchial secretions.

The role of air pollutants in relationship to the incidence of bronchial epithelial hyperplasia as related to the above program.

5. Detailed Plan of Procedure:

See attached material.

6. Budget Plan:

(See page 3)

a. Salaries	\$ 17,800.00
b. Expendable Supplies	6,100.00
c. Permanent Equipment	4,500.00
d. Overhead (15% of a & b)	3,600.00
e. Other (travel)	1,000.00

Total \$ 33,000.00

7. Anticipated Duration of Work:

Three years.

8. Facilities and Staff Available:

See attached material for these items.

9. Additional Requirements:

10. Additional Information (Including relation of work to other projects and other sources of supply):

/s/ A. H. Stein, Director of Project  
/s/ R. J. ? Asst. Treas.

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At the Surgical Forum, 45th Clinical Congress, 1959, we reported on the induction of bronchial epithelial hyperplasia by human cancerous bronchial secretions. Two series of preliminary experiments were reported. In the first experiment, bronchial aspirates were obtained at the time of bronchoscopy from two patients with carcinoma of the lung. These patients had tracheostomies and repeated bronchial aspirations were made until 100 ml. of aspirate was collected. This material was then lyophilized, re-suspended in 100 ml. of isotonic saline, and filtered through a Seitz bacterial filter. A similar procedure was followed in 12 patients who were known to have pulmonary emphysema. These patients were bronchoscoped and the bronchial secretions were obtained. It required a total of 12 patient collections to again obtain a 100 ml. volume as the noncancerous control.

Thirty male hamsters about  $4\frac{1}{2}$  months old were divided into three equal groups. One group received saline injections; one group received saline with nonneoplastic aspirate; and one group received saline with neoplastic aspirate. The injection program entailed 0.5 ml. subcutaneously twice weekly for 22 weeks. The animals were then sacrificed. The bronchial tree and other organs were carefully dissected out and fixed in formalin. The bronchial tree was embedded en bloc and step sections were made. Histologic observations revealed polyp formations in the bronchi of 7 of the 10 hamsters which had received injections of the neoplastic material. Neither of the control series showed such formations. The remainder of the organs showed no abnormalities.

Second experiment: In the second experiment endobronchial aspirates at the time of bronchoscopy were obtained from 5 patients with carcinoma of the lung and 4 with pulmonary emphysema. Each sample consisted of approximately 5 to 10 ml. of aspirated material and each was processed individually, as in experiment 1. The samples were lyophilized, re-suspended in tissue culture medium, and passed through a millipore bacterial filter. A portion was then set aside (frozen) as a noncultured control. Human serum was then added to the remainder and this was divided into two portions, one used as a gross medium for S-3 hela cells, the other for FL-amnion. These cultures were grown for 9-10 days, then removed from their bottles and disrupted ultrasonically. Similar groups were pooled. The only abnormality on short-term tissue culture was observed in the FL-amnion neoplastic cultures which showed some cytopathogenic effect after 3 days. Ninety male hamsters, 5-7 weeks old, were divided into six groups of 15 each. Hela, FL-amnion and noncultured; each in a neoplastic and non-neoplastic series. The injection program was the same as previously but was continued for only 7 weeks. The histologic preparations were identical. Again, the hela neoplastic series yielded a 45 percent of polyp formation, the noncultured neoplastic series, a 51 percent incidence and the amnion cultured neoplastic series, a 70 percent incidence. The correlation between the cytopathogenic effect in FL-amnion and the increased incidence of polyp formation in this series was suggestive of a potentiating effect.

Experiment 3. Currently experiment 2 has been repeated in a large enough series to be statistically valid. Furthermore, at the time of sacrifice the bronchi are washed with saline and aspirated. We will attempt to complete Koch's cycle in order to strengthen our opinion that a factor is truly present in the secretions of human cases of bronchogenic carcinoma which can be transmitted and ultimately recovered.

1003541255

Proposed study: We feel that there is evidence that in the secretions of patients with bronchogenic carcinoma there is a factor which specifically stimulates bronchial epithelium to undergo hyperplasia, even when this material is injected in a distant site. We propose to evaluate the role of various air pollutants in relationship to the incidence of bronchial epithelial hyperplasia as related to the injection program described above.

Currently we feel that the anatomic knowledge that

- a) 90 percent of carcinomas arise in the region of the hilum
- b) multiple in-situ lesions occur in association with carcinoma of the lung
- c) there is relative freedom of the tracheal epithelium suggesting that a factor is secreted by the epithelium or peribronchial glands and in the physiologic movement of secretions to the hilum where they are progressively concentrated.

The maximum concentration should be topically in the region of the hilum. Subsequent to the cough reflex the material is brought up without direct application to the tracheal mucosa. It may be that the effect of air pollutants is direct only in that they alter the chemical character of the secretion, the volume of the secretion and the concentration.

After concentration we propose to inject a series of mice and hamsters, newborn and weanlings, with and without steroid, with the cell-free extract from bronchial secretions of patients with and without bronchogenic cancer. Half of the animals will be subject to periods of smoking and half will remain as controls. It will be of great interest to see whether the bronchial epithelial irritation associated with air pollutants has any effect quantitatively or qualitatively in the nature of the lesions which we have previously described. At this time I might again indicate that the lesions described in our previous experiments were observed without any inflammatory cell response.

As another type of control experiment we propose to reduce our cell-free preparation to a powder, reconstitute them with saline and then create a spray effect which the experimental animal may inhale. This would give us information in regards to the topical effect of bronchial secretions in relationship to the respiratory epithelium.

Subsequently, we will attempt to fractionate our preparations from patients with bronchogenic carcinoma in order to identify the particular chemical fraction which appears to be responsible for bronchial epithelial changes.

The next step in this program will be to separate chemical fractions in the prepared cell-free materials from controls and patients with bronchogenic carcinoma. Chemically our efforts will be directed towards the separation of proteinaceous, nucleic acid or virus-like material. On the basis of physical properties various fractions could be separated and evaluated in relationship to associated injection experiments.

1003541256

## Budget:

## a. Expendable supplies--

Animals	\$ 4,000	
Glassware	1,000	
Filters	300	
Chemicals	<u>800</u>	
		\$ 6,100

## b. Permanent equipment--

Animal cages	500	
Freeze-dry unit	1,500	
Bank of smoking machines	1,500	
Fraction collector	<u>1,000</u>	
		\$ 4,500

c. Other-- Travel to scientific meetings	<u>1,000</u>	1,000
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## d. Personnel--

Animal care	3,600	
2 tissue technicians	}	
1 biochemical technician		
@ \$4,000	12,000	
1 part-time secretary	1,200	
2 part-time medical student assistants	<u>1,000</u>	
		17,800

e. Overhead -- 15% of a and d	<u>3,600</u>	3,600
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Total		<u>33,000</u>
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Facilities and staff available-- My Curriculum Vitae is enclosed. Dr. Michael Vanko (Ph.D), Instructor, and Director of the Clinical Laboratories at the Albany Hospital will be basically in charge of the development of the fractionation and chemical analyses of the prepared material. Joseph V. Landau, Ph.D., Research Associate in Oncology, will be primarily responsible for the preparation of the bronchial secretions. Allan Stranahan, M.D., Associate Professor of Thoracic Surgery, is cooperating by providing us with all the material from patients who are bronchoscoped in his very active department.

Anticipated Duration of Work: The program is planned for a three-year period. The budget for the first year has been outlined on page 5. We feel that in the next two years a budget of \$24,000 annually will be necessary.

1003541257



Committee:  
Dr. Wilson  
Dr. Little  
Dr. Cattell  
Dr. Bing

TOBACCO INDUSTRY RESEARCH COMMITTEE

150 East Forty Second Street

New York 17, N.Y.

Date: April 25, 1960

#275  
(cf. #89  
Activated: 10/1/55  
Renewed 9/1/56  
#168  
Activated: 9/1/57  
#203  
Activated: 9/1/58  
Renewed: 9/1/59

1. Name of Investigator: Caroline Bedell Thomas, M.D.
2. Title: Associate Professor of Medicine
3. Institution & Address: The Johns Hopkins School of Medicine  
725 North Wolfe Street  
Baltimore 5, Maryland
4. Project or Subject:
  - a. Completion of Studies on Smoker-Nonsmoker Differences among Johns Hopkins Blood Bank Donors.
  - b. Continuation of Studies on Psychological Smoker-Nonsmoker Differences among Johns Hopkins medical students.
  - c. Continuation of Studies on the Ballistocardiographic Smoking Test.

5. Detailed Plan of Procedure:

As discussed in the accompanying Progress Note (attachment 1), the work projected under our current two-year TIRC grant is not yet complete. Therefore, a grant for an additional year is requested for the following purposes:

a. Completion of Studies on Smoker-Nonsmoker Differences among Johns Hopkins Blood Bank Donors. It is proposed to write two more papers on smoker-nonsmoker differences in these healthy subjects: the first, a comparison of cholesterol levels and the second, a comparison of parental smoking habits, causes of parental disability and death, along the lines of the first two papers of the series (attachments 3 and 6).

b. Continuation of Studies on Psychological Smoker-Nonsmoker Differences among Johns Hopkins Medical Students with Particular Reference to Figure Drawings (Draw-a-Person Test). After a number of exploratory studies (see attachment 11) and after finishing the "Review of the Literature" now in the process of completion and making suitable reproducibility checks, it is our purpose to write at least two papers analyzing our figure drawing material with especial attention to sexual differentiation and identification and the relationship of body type to self image. The results of these studies will then be available for comparison with the anthropometric measurements of sex components and body type now being made by Dr. Carl Seltzer on the same Johns Hopkins medical students under an independent TIRC grant.

c. Continuation of Studies on the Ballistocardiographic Smoking Test.

1. Much of our tabulated data on ballistocardiographic form before and after smoking is not yet published. A new paper is projected in which the points touched on at the end of the New York Academy of Sciences paper (attachment 5) will be expanded and other data added.

1003541258

2. At the recent New York Academy of Sciences Conference, Dr. J. H. Burn of Oxford, England reported that nicotine produces cardiac acceleration through the release of local stores of adrenalin and noradrenalin, and that this effect is neutralized by reserpine. It is proposed that exploratory studies be made using reserpine, along the lines of our hexamethonium and amphetamine studies, to see how the individual patterns of circulatory response to smoking in man may be altered. This approach might provide a rough index or indirect bioassay of human stores of catecholamines.

6. <u>Budget Plan:</u>	Salaries	\$ 8,550*
	Expendable Supplies	500
	Permanent Equipment	---
	Overhead (15%)	1,500
	Other (Tabulation and employee welfare benefits 5%)	950
		<hr/>
		\$11,500

7. Anticipated Duration of Work: One year

8. Facilities and Staff Available: a. Facilities: There is ample space available at our research project headquarters. b. Staff: 1) Dr. Caroline B. Thomas devotes full-time to the combined research projects (TIRC and N.H.I. grants). 2) Dr. Leona Wise Jones, Research Psychologist, joined our staff on a two-day a week basis on Dec. 1, 1959 to analyze the figure drawing material. She will be available on the same basis during the coming year. A recent arrival in Baltimore, she taught Educational Psychology and Educational Tests and Measurements at the Johns Hopkins Univ. last summer. (Ph.D. in Psychology at Northwestern Univ. 1944; has held a series of full-time teaching and administrative positions; Who's Who in America, 1958-59, American Men of Science III, Social and Behavioral Sciences). 3) Dr. Bernice Cohen, collaborator in the Blood Bank studies, is a Post-Doctoral Fellow in Genetics (Chr. Dis. Div., J.H. Sch. of Hyg. and Publ. Health and Div. of Med. Genetics, Dept. of Med., J.H. Sch. of Med.). 4) Dr. Kendrick joined our staff Sept. 1, 1959, as full-time statistician under a supplementary grant from the N.H.I. 5) Dr. E. W. Slockbower, clinical psychologist, administers and interprets psychological tests.

9. Additional Requirements:

None

10. Additional Information (Including relation of work to other projects and other sources of supply):

The aims of the project outlined above are in harmony with those of Grant H-1891 (C6) entitled "Precursors of Hypertension and Coronary Artery Disease" awarded by the National Heart Institute. The funds from that source do not include most of the items covered by the budget given above. Where similar items exist in each of the two budgets, it is because the budget from Grant H-1891 (C6) is insufficient to meet the total expense of a given item, and the two budgets will be used in such a way that they supplement each other.

1003541259

\* See next page.

\*Breakdown of Salary Request:

Director, Dr. Caroline B. Thomas	\$ 3,000	(she receives \$5,500 from N.H.I.)
Research Psychologist, Dr. Leona W. Jones	2,400	
Statistical clerical and secretarial	3,150	
	<hr/>	
	\$ 8,550	

/s/ Caroline Bedell Thomas  
Director of Project

/s/ Thomas B. Turner  
Business Officer of the Institution

1003541260

## TOBACCO INDUSTRY RESEARCH COMMITTEE

150 East Forty Second Street

New York 17, N.Y.

Application For Research GrantDate: November 1, 1959

1. Name of Investigator: Sam I. Stein, Ph.D., M.D.
2. Title: Coordinator of Research and Medical Director
3. Institution & Address: Bertram & Roberta Stein Neuropsychiatric Research Program, Inc.  
6770 N. Lincoln Avenue, Lincolnwood 46, Ill.  
(In collaboration with Gerhard Closs, Ph.D., Assistant Professor, Department of Chemistry, University of Chicago.)
4. Project or Subject: See attached statement.
5. Detailed Plan of Procedure: (a) Staggered dosages of oven dried mushrooms which have already been tested for their psychoneuropharmacological effects (*Panaeolus venenosus*, *Panaeolus sphinctrinus*, and *Psilocybe caerulescens*) will be administered in increments of one-half gram amounts to selected subjects.  
  
(b) The subjects will be non-patients and also patients drawn as volunteers from a neuropsychiatric practice.  
  
(c) The full pharmacological mushroom effect is known to last on an average of two hours. A cigarette of patient's choice will be offered on two occasions during each experiment. The effect of the cigarette will be compared with two categories of stimulants given orally in solution, (a) caffeine sodium benzoate and (b) amphetamine sulphate; the effect will also be compared with variable dosages of niacin (nicotinic acid) given enterally and parenterally.  
  
(d) The measurements to be made before, during, and perhaps after the experimental process will be:
  - (a) Subjective statements of patients.
  - (b) Examiner's observation of patient's conduct.
  - (c) Temperature, pulse, blood pressure, pupillary size, and skin texture or status at regular intervals.
  - (d) Draw-A-Person test.
  - (e) Polygraphic studies (Stoelting apparatus) at significant intervals.
  - (f) Metabolites in blood and urine such as are routine but pertinent; but also special studies pertaining to general metabolic state (cholesterol, protein-bound-iodine), to liver function (transaminase), and to niacin degradation.
  - (g) Some tape recordings of patient's statements.

1003541261

6. Budget Plan:

Salaries	\$ 7400.00**
Expendable Supplies	500.00
Permanent Equipment	6000.00
Overhead	1200.00
Other	1200.00*
	<u>\$ 16300.00</u>

7. Anticipated Duration of Work: One year

8. Facilities and Staff Available:

- 1 - Sam I. Stein, Ph.D., M.D. Chief Investigator
- 2 - Jerry R. Hora, M.D., M.S. \* Associate Investigator (part time)
- 3 - Jesus de la Hueraga, M.D., Ph.D.\* Biochemist (Consultant)
- 4 - Gertrude B. Stein, B.S. Laboratory technician
- 5 - Polygraphic equipment (Stoelting) now available
- 6 - Space for research in the offices of Dr. Sam I. Stein, 6770 N. Lincoln Ave., where two days per week are reserved for research activity.

9. Additional Requirements:

- 1 - Additional part time will be required from these two members for this project \*
- 2 - Full time research technician \*\*

10. Additional Information (Including relation of work to other projects and other sources of supply):

(a) The study proposed above regarding specific observations of the effect of cigarette smoking (nicotine) is part of a large program designed to examine the effects of significant mushrooms on the nervous system. The ultimate goal of this program is to seek pharmacological products which might be helpful in expediting 'neurosis-therapy', that is, if possible, to create an elevated functionality in the nervous system to replace the inhibited or sub-potential functioning as obtains in neurosis.

(b) The larger Program is affiliated with the Chemistry Department, University of Chicago, (through Prof. Closs; with the Pharmacy School, University of Illinois; and with the Mycology Research Department, Penn State University, (Dr. R. Kneebone).

(c) It may be incidental that Dr. Hora and I (Stein) have theories of malignancy which exclude causation such as the one correlated between smoking and lung cancer. Dr. Hora has worked and published on the idea that psychological-emotional states are pertinent in malignancy causation. My own theme (Stein) of malignancy causation is based on the fundamental irritability (as defined in biochemical and metabolic terms) threshold(s) of the nervous system. I believe that a chemo-therapeutic approach is needed but directly to the nervous system to prevent malignancy evolvment and not after the fact of malignancy development where the task of seeking chemical antidotes for each malignancy appears unrealistic. Of course, this malignancy feature is not an immediate part of our investigations but the concept is always held in mind as the effects in other studies are being observed.

/s/ Sam I. Stein, M.D.  
Director of Project  
/s/ J. Salomask?  
Business Officer

1003541262

STATEMENT ON PROJECTED STUDY

to be submitted to

TOBACCO INDUSTRY RESEARCH COMMITTEE

By

Sam I. Stein, Ph.D., M.D.

Medical Director, Bertram and Roberta  
Stein Neuropsychiatric Research Program, Inc.  
(In collaboration with Gerhard Closs, Ph.D., Assistant  
Professor, Department of Chemistry, University  
of Chicago.)

Experiments have been in process with "hallucinogenic" mushrooms administered to human subjects. Mixed effects have resulted and tentatively reported; (1), (2), (3). Separate investigations are being made to determine the clinical effects of an amino acid (L-tryptophane) and of vitamin B-complex. The probable correlation between the several experimental products is being studied primarily from the neuropsychopharmacological aspect. Some tentative correlations have been reported by one of us (see abstract of presentation at AIBS meeting, September 1959) where an emphasis upon niacin metabolism is made.

In the course of a mushroom experiment with one of us the subject (G. C.) and the other the observer (S.I.S.), where a rather prominent and uncomfortable parasympathetic (serotonin-like) stimulation seemed to occur (p. 48; b.p. 98/70; temp 97<sup>0</sup>, cold extremities), the smoking of a cigarette at this point of the situation immediately gave a favorable type of subjective relief with reading changes reaching to p. 58 and b.p. 108/65. Within a few minutes after the cigarette was smoked the readings returned to p. 48 and b.p. 104/70, and with the slowed down effect came the subjective comment, "I feel pretty miserable now". Another cigarette later in the test process produced less marked but comparable effects. In contrast to the cigarette, the stimulant, caffeine sodium benzote, when administered tended to reduce the pulse and blood pressure to lower levels p. 44 and b.p. 100/60 respectively.

Our intent is to determine the chemical substance in the mushroom which is causing the parasympathetic stimulation. It is evident that the cigarette smoking here contributed a desirable stimulatory effect and had offset the "slowed-down" physiology and feeling which occurred with the excessive parasympathetic stimulation.

It is our opinion that the nicotine of the cigarette was responsible for the enhancement of physiology and of feeling-tone observed here. Is this a more pronounced aspect of a mechanism or situation which obtains in the stimulatory effects arising generally in cigarette smoking? Is this a basic phase of the explanation for the latter? What relationship does nicotine have to niacin in the general stressor (Selye) or nervousness process? One of us (S.I.S.) contends that the sympathetic (with adrenalin-noradrenalin-histamine) part of the autonomic system produces the symptom-signs and feeling-tone of "nervousness" and the parasympathetic (acetylcholine) produces the symptom-signs and feeling tone of non-nervousness (slowed or quieting effect); whereas, the components of mood

1003541263

(euphoria versus depression) and associated energy are correlated with the status of the reticular (alerting) formation of the brain stem and is subserved by a phase of niacin metabolism.

In the further experimentation with the mushrooms or the chemical isolated from such and with L-tryptophane, the cigarette (nicotine) could be used as above in a further study of the observed effect as well as to determine the probable correlation with niacin metabolism. The further experimentation therefore should include laboratory examination of at least the niacin metabolites as they may be detected in the blood and the urine (Determined according to the methods used at the University of Wisconsin in their studies on L-tryptophane-niacin-serotonin (4).)

1. Observations On Agarics Causing Cerebral Mycetisms, Rolf Singer, Dr. S. I. Stein, Dr. Ralph W. Ames, and Dr. Alexander H. Smith. *Mycopathologia et Mycologia Applicata* - Vol. IX, 29-IX-1958, Fasc. 4.
2. Some Clinical and Chemical Observations Of *Panaeolus Venenosus*, *Panaeolus Sphinctrinus*, and *Psilocybe Caerulescens* Mushrooms. Sam I. Stein, M.D., Ph.D., *Mycologia*, Vol. L-1, January-February 1959.
3. Observations On Psychoneurophysiologically Significant Mushrooms. Sam I. Stein, Gerhard L. Closs and Norman W. Gabel. In press, *Mycopathologia et Mycologia Applicata*.
4. Disorders Of Tryptophane Metabolism. J. M. Price, University of Michigan Medical Bulletin, Vol. XXIV, pp. 461-485, 1958 (Dec.).

1003541264

ABSTRACT

Some Biochemical and Physiological Correlations Developed From Clinical Observations With Various Toxic Mushrooms and Medicinal Products.

Sam I. Stein,\* Ph.D., M.D.

Clinical and some psychological measurements have been made after the ingestion of graduated amounts of *Panaeolus venenosus*, *Panaeolus sphinctrinus*, and *Psilocybe caerulescens* mushrooms. These are compared with observations derived in clinical practice resulting from the application of psychopharmacological products (reserpine, iproniazid, vitamins, and some amino acids). Some practical correlations appear permissible at this time. Recurring effects and some variations will be presented. The latter might be explained on the basis of individual biochemical differences.

\* Sam I. Stein, is Medical Director, Clinical Investigator and Coordinator of Research of The Bertram and Roberta Stein Neuropsychiatric Research Program, Inc.; and also a private practitioner in neuropsychiatry.

The full paper is to be published in the Proceedings of the Society of Industrial Microbiology, 1959.

1003541265



SOME BIOCHEMICAL AND PHYSIOLOGICAL CORRELATIONS  
DEVELOPED FROM CLINICAL OBSERVATIONS WITH VARIOUS  
TOXIC MUSHROOMS AND MEDICINAL PRODUCTS

Sam I. Stein, Ph., M.D.

Perhaps, first, I should establish a rationale for having a neuropsychiatrist on the program of industrial microbiologists. Individuals in your category must focus a sharp scientific eye on very small things, and the other eye might be focused on the idea of practical application. In fact, initially, it may take the fullest or complete focus of your attention to a small biological sphere or area before the mind's eye turns to classify the observation into its correct place in the larger biological scheme. Contemporary psychiatry would do well to (or must) pass through a similar 'micro' orientation regarding the fundamentals of its material.

Terminology is always important in science. The term psychiatry is misleading. It implies an emphasis on psyche or mind. But the mind in the neurological or physiological sense is only another variant in the fundamental functioning of a neural system. True the mind has a special significance or importance in that its specific content is coded into the human individual as its life period progresses. But for the psychiatrist to place a main emphasis on the quality and quantity of the mind-content makes him only a competitor with the social scientist, the educator, the psychologist, the clergyman, the sociologist. To presume that the properties of the organic and physiological substrate of the mind in the nervous system are always constant is to overlook the more significant feature of potential instability in the entire sequence in the complex of mind functioning. Grundfest in "Evolution Of Conduction" states, "The rapidly expanding organizational and functional complexities of the nervous system arising from mere increase in their number accompany and in turn make possible the expanding capacities of animals in different evolutionary stages to cope with their environment. One may hope optimistically that Homo sapiens of future generations will be endowed with a more highly developed and better functioning nervous system than are his current progenitors". To most of you who are not active in my field of work I may not have the problem with psychiatric preconception. I hope that I made my point clear that in psychiatry an emphasis of observation is needed to man's internal environment, i.e., the mind's organic and physiological substrate, how these latter integrate with the fundamental functions of the nervous system and in turn the functional interdependence of the nervous system with other systems of the body, most significantly with the metabolic.

Now, I should like to approach my observations of mushrooms with the following clinical analogy. If a drop of dilute atropine is placed in the conjunctival sac, the pupil of the eye dilates within a few minutes. Here is a situation in clinical practice where the ophthalmologist produces a relatively local effect with one specific chemical, hopefully, upon one or a specific nerve which anatomically is so located as to make this technique feasible. I said, "hopefully upon the one nerve" because the atropine is also absorbed into the local tissue fluids from whence it enters the systemic circulation. Because even the few drops needed to dilate the

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pupils and even when applied to a serous membrane type of tissue rather than the usual routes of medicinal administration, produce in some undesirable systemic effects (palpitation, dry mouth, disturbed intestinal activity), the diluted atropine is being replaced by even more dilute products capable of dilating the pupil but not as active upon other tissues if absorbed systemically. Please, note how many observations can be derived from or how many variables must be considered in this one situation most of which are pertinent to the clinical study of all neurophysiologically significant pharmacological materials. Among these variables of consistent consequence are the factors of dosage, avenues of absorption, local effects, systemic effects, undesirable side-effects, time required for induction of effects, and the variability of response of the individual human. In psychiatry, in addition, the subjective feeling tone arising in a complex situation, such as having some "burning" drops placed in the eyes at a doctor's office by personnel of variable attitudes, is of consequence in the measurement of drug effects since the subject's stressor mechanism may become activated in the situation to produce complicating effects from the sympathetic-autonomic apparatus. Perhaps in some instances the tension-reaction, that is the anxiety, is so great that the atropine is almost unnecessary to dilate the pupils. An additional item within the feature of control in this type of clinical test observation is the observer's ability to be objective, and here again semantically correct in his reporting. Returning to the subject with his pupils now dilated, one could describe him as "wide-eyed" or "wild eyed". The former expression intends to describe the status of the pupils only, whereas, the latter implies in addition an observation as to the affective, attitudinal or mental state of the subject. From this former example of a specific fairly well known chemical applied by a specific route, if one keeps in mind some of the tangible and seemingly intangible variables to be measured, let us turn and compare it to a situation where a human individual has taken a bite of food, let us say meat or perhaps mushroom. Now we have a condition where a number of specific chemicals or prechemicals are brought into the organism by another even natural route. The variables to be considered may not be more numerous but each variable becomes more complex inasmuch as the time factor in securing effects depends upon almost the entire complex of metabolism in general, upon the special process of some particular chemical, the additional complication of the interaction between the metabolites formed from the bite of food, and interaction between the newly processed metabolites present with those in the body's pool of such substances. Fortunately, much is known about the metabolic process. Perhaps most of it was learned from the usually species-fixed metabolism of the subhuman animal, but much of it applies to man. Whether this is also true regarding the human nervous system is controversial. In my opinion, in this most important area, namely, the controlling effects of and the needs of the nervous system, that is, the neurally affected metabolism in man contains either primary or potential significant differences and/or variations of pathways from those of the infra-human form. The latter may become so extreme at times that they might be considered under the term errors of metabolism or metabolic errors.

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All of the preceding has been presented in the hope that it will serve as a background to my discussion of mushrooms. Actually, my statements here are not so far removed to that of mainly trying to find a method to study and to understand the effects of some mushrooms as observed in the human. There is an extensive literature rapidly accumulating on the history of the mushroom's place in medicinal practices. This history dates as far back as there are intelligible records. Some of the reports and claims that have been expressed as to the effects of mushrooms on man's mind and/or behavior are so unusual that they appear imaginative. This is a topic of its own and I don't have time for it here. Besides, my own interest in neuropharmacological mushroom effects did not arise from an outside source of stimulation but mainly by chance in 1949 when I personally became involved in an unusual experience that occurred after a meal which included apparently cultured mushrooms. A subsequent study of the literature verified my inference that the phenomenon which I had experienced and observed was most probably precipitated by the mushrooms in that particular meal. But I didn't come here to tell you of the concepts or speculations I had developed about mushrooms from that experience. Having had formal graduate study in medicine, I continued to apply the scientific method in my medical practice. Following the above experience, I gradually shifted my research to the investigation of mushrooms. This is clinical research of the human which probably involves the most complex complexes of neuroid substance and of metabolism. I said, "gradually I shifted my research" because prior to approximately 1952, it would have been considered irrational or complete heresy to imply that symptomatic adjustments in psychiatric material could be secured even by standard drug or chemicals. So prior to that time, to have made any favorable psychiatric claims for toxic mushrooms might have cost one his membership in organized psychiatry if not even his medical license.

Through a sequence of correspondence and personal contacts which started late in 1951 with Dr. A.H. Smith of Michigan, I met Dr. Rolf Singer in 1953 at the Chicago Museum of Natural History. Dr. Singer verified my inferences as to the neuropharmacological effects of mushrooms, particularly the genus, *Panaeolus*. Nevertheless, it took several years before I was able to develop an organization to sponsor such a study. In 1955 our group became active and soon thereafter, I discovered that another group consisting of the two Wassons and Dr. Roger Heim, the mycologist, were also investigating similar effects of mushrooms among the Mexicans which situation had come to their attention as the lately deceased Mrs. Wasson, a physician, was pursuing her avocational interest in mushrooms.

My group made contact with Dr. Ralph Kneebone, who as you may know, does research in the growing of mushrooms right here at Penn State University. Upon consulting with Drs. Smith and Singer again in 1957 it was decided to explore the *Panaeolus venenosus* species since this mushroom was the most likely contaminant of the cultured variety. It was known to have caused unusual central nervous system effects in instances where it was accidentally ingested. Happily, Dr. Kneebone succeeded in growing a sizeable amount of *Panaeolus venenosus* in his first try. Dr. Singer was despatched to Mexico to secure the significant mushrooms there which were intended to be used comparatively to our studies of *Panaeolus*. In addition, Drs. Kneebone

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and Singer were able to grow the Mexican varieties of the genus, *Psilocybe*, here at Penn State so that we were assured of a constant and sufficient supply of this material.

I am prepared to report in part three separate items of observation derived from the use of our mushroom material. The first item is one already to be found in the literature and consists of my taking approximately 5 grams of oven dried *Psilocybe cubensis*, fried in butter. For whatever it may be worth scientifically, a tendency has developed in the neurotropic-drug-investigations, for the investigator to sample his own soup, so to speak. I had already tried various amounts of *Psilocybe* material before. In my adventure with *cubensis* on a particular Sunday in December 1957, I was seeking to determine whether a comparable effect would or could be produced to the one I had experienced in 1949, the one which came my way serendipitously. First, I want to report that essential theme of my *cubensis* experience is one in which I was as close to feeling dead as I ever want to be, but actually for a period of nearly six hours I felt sicker than at any time in my life. Unfortunately, very few objective measurements could be made on myself since I was completely unprepared for what had evolved and transpired. I can tell you that unhappily for myself, my mind remained clear, but I believe that whatever hallucinating or disorganization of mind that is alleged to occur as a result of the *Psilocybe* substance was taking place in all other parts of my nervous system and other tissues. My skin was so anesthetized that I was unable to feel my own pulse. Literally, it appeared as if both the sympathetic and parasympathetic parts of my autonomic apparatus were doing battle at various times. Presumably, the sympathetic component emerged victor, if the saucer-like size of my pupils were to be used as a main criterion. I believe I have personally tested every drug that has been deposited in my office by the pharmaceutical's salesman. But for certain, I have tested some of those being used in neuropsychiatric investigation such as mescaline and lysergic acid diethylamide, which are other so-called hallucinogens. Here I experienced the standard effects I had observed or had been reported by others. In view of my *cubensis* observations, I proceeded cautiously or more judiciously in the experimental application of our whole mushroom material. The dried products were granulated and weighed in  $\frac{1}{2}$  gram amounts. I decided on this dosage-size on the basis of weighing such an amount of whole, dried, Mexican mushrooms as was reported to produce a pharmacological effect among the natives in Mexico. Of the three items to be described here, in contrast to the first, namely, my *cubensis* experience with its relatively random approach, the following two which are separate case studies might be viewed as systematized, if they are considered in the perspective of clinical psychiatric practices.

The first of these cases might be summarized as follows:  
My patient, J.H., age 27, had been under my care since April 13, 1958. Because of homosexual thoughts, rapid pulse (112), and a moderate hypertension (165/90), he was on the following medication (May 20) designed to control his symptoms:

Reserpine - conc. sol., 16 drops qid  
Iproniazid - 25 mg. tablet,  $\frac{1}{2}$  tablet bid  
Amphetamine - 5 mg. tablet,  $\frac{1}{2}$  tablet  
Amytal - grain  $\frac{1}{2}$ , 1 bid

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On May 4, the subject had reported a clearing of his homosexual thoughts; and by May 6, he could not restore these thoughts even if he tried. On May 6th his clinical readings were: blood pressure 120/70, pulse 72, and weight 126. The Draw-A-Person test\* was used almost daily to validate his subjective claims. Although homosexual thoughts were inactivated, the positive heterosexual thoughts were asserting themselves only slightly. The subject was from Ireland via Canada, and his visa time was becoming short. He had read of my work with mushrooms, and had originally come to Chicago in the hope that mushroom effects would help. He volunteered spontaneously to try the crude material in hope of expediting the effect he was seeking. On May 20, while still on the medication listed,  $\frac{1}{2}$  gram *P. venenosus* was given without significant effect. At intervals of 2 or 3 days more experiments were attempted. One gram produced positive results. One and one-half grams produced strong favorable effects. *Psilocybe caerulescens*, one and one-half grams, was also tried, but a different, relatively hallucinogenic, and much less pleasant effect was secured or reported. The *panaeolus venenosus* effect was only minimally hallucinogenic and only in the one and one-half gram portions. There was no doubt from these observations that the *Panaeolus venenosus* contained psychoneurophysiologic ingredient. Its effect was clearly different than the *Psilocybe*. Its effect was mainly stimulatory (euphoriant) and no undesirable side effects were reported or noted. The subject asked for more of the effect which was secured with one and one-half grams of the *Panaeolus*.

I have rushed through the findings in this latter case since I believe the material of the succeeding study not only is more extensive but the subject was a non-patient and his findings were not complicated by any pre-medication. In fact, the subject to whom I now refer is Assistant Professor Gerhard Closs of the University of Chicago who is collaborating in our research activity and who offered to be the figureative "guinea pig" since he wanted to experience personally the effects of the chemicals he is seeking to isolate and identify. I had invited him to come to this meeting as a discussant to give you personally his various observations and inferences, but unfortunately he had earlier plans to be on the West Coast at this time.

As these case studies proceed, I have been making the following measurements:

- a. Weight of subject
- b. Oral temperature at variable intervals
- c. Pulse, blood pressure, and sweat reactions at 10 to 15 minute intervals
- d. Estimated pupillary size and reflexes at frequent intervals
- e. Patient's subjective feeling-tone statement is sought (almost as often as the pulse reading)
- f. My observation of the subject's reaction is continuous since I never leave the subject after he partakes of the mushrooms until the evidence indicates that the experimental process has stopped.

\*Slide no. 1 and no. 2

Before I give you a summary of my findings in this case study, I might present a few charts taken at significant points of these experiments. The time will not permit covering all the steps of even one experiment since the observations cover from 2 to 5 hour periods. However, some of this material has been in press since March, and I hope that the journal will be publishing it soon.

My summary of the findings to the present time reads as follows:

There were noticeable similarities and differences in the effects of *Panaeolus venenosus* and *Psilocybe caerulea* which appeared to have no correlation with the quantity of raw mushroom ingested.

In the experiments conducted so far, the subjective and objective effects developed within about the same time interval after ingestion, 25 to 30 minutes, with one exception. Namely, yawning, which was observed here and elsewhere only as occurring with the *Psilocybe* material, usually within 5 to 10 minutes, and it proceeds until other symptoms and signs present themselves.

Reported symptoms or subjective effects which occurred with *Panaeolus* only or mainly were (a) a mild or moderate relaxing (tranquil) type of inebriation, (b) disturbed equilibrium, (c) either diminished motivation or blocking in the psychic (thought) process, and (d) paresthesias.

Features which obtained with *Psilocybe* only or mainly were (a) the aforementioned yawning in the induction period, (b) "burning" sensation in the esophageal and stomach areas, (c) a feeling of extreme tiredness, and (d) a feeling-tone of anxiety bordering on collapse associated with a sense of strong intoxication.

The factors occurring in common were (a) the extreme reduction in the pulse rate, slightly more with *Psilocybe*, (b) the steadiness of the systolic and diastolic components of the blood pressure with both varieties of mushrooms, except for a very brief period of drop in both diastole and systole with both *Panaeolus* and *Psilocybe* during the period of strong intoxication, (c) dilatation of the pupils (d) a drop in body temperature, seemingly more marked with *Panaeolus*, but productive of much more subjective effect (feeling of cold) during the period of *Psilocybe* intoxication and (e) sweating.

With *Panaeolus* there was a general drop in the total personality integration, but the internal sensation was one of feeling pleasant and relaxed; whereas, with *Psilocybe* there was actually disorganization associated with the feeling of panic and disagreeable intoxication.

A most significant feature is that Dr. Closs at no time perceived or reported effects that could be categorized as hallucinatory. However, in my first subject a quasi-hallucinoses developed but more with the thought processes rather than with colors. In my own *cubensis* experience, I had observed strange color effects and disturbed stereognosis.

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My formulation as to the meaning of these observations reads as follows: Obviously, it is unwise and perhaps unnecessary to try to correlate clinical observations where unknown chemicals or chemical complexes are ingested in combination.

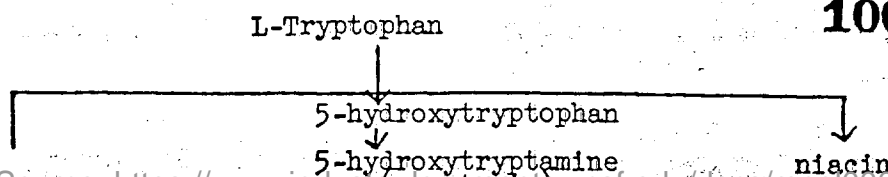
But since I have had a considerable experience with psychoneuropharmacological (psychoneurotropic) material and also with the literature of biochemistry, I offer the following tentative opinion. The relaxing subjective effects, the hypothermia, and diminished pulse found with *Panaeolus* resembles the effects attributed to serotonin. But the inebriation and the pupillary mydriasis probably cannot be explained as effects of serotonin.

Some of the effects observed here (and elsewhere) with *Psilocybe* are probably due to the serotonin-related-*psilocybin* already isolated from the *Psilocybe mexicana* (Heim) which substance probably also obtains in the *Psilocybe caerulescens*, the species used in our experiments. Again however, it seems unlikely that *Psilocybin* itself will be found to have caused the signs of mydriasis and of cerebral or cortical intoxication. Where might one turn for some reasonable explanation to this variety of effects? Is there an orienting concept that one might develop to serve as a guide to the present information and to what may evolve? Our *Panaeolus* mushroom as yet has not been chemically analyzed. I have Dr. Closs's paper chromatographic spread to show. (Slide no. 3)

I shall not attempt to interpret his findings but will read his summarizing paragraph of our combined paper which is in press:

"The extracts of *P. venenosus*, *P. sphinctrinus*, *Psil. mexicana*, *Psil. cubensis*, and *Psil. caerulescens* have been compared by paper chromatography. It was found that neither *Panaeoli* contain *psilocybin*, the main constituent of the three *Psilocybe* species. The extract from *P. venenosus* has been chromatographed, and two of the three major constituents were purified and obtained crystalline. One of these compounds could be shown to possess the same chromophore as *psilocybin*, a 4-oxygenated indole system, and seems most likely to be the active compound of the mushroom".

Since our material has not been isolated and identified in a final chemical way, I shall proceed with my tentative formulation of trying to explain clinical results by turning to the known chemical structure of *Psilocybin* (slide no. 4) which you will see when hydrolyzed yields 4-OH-Tryptamine instead of 5-OH-Tryptamine which is serotonin. Before proceeding further, I should like to bring to your attention some chemical and clinical observations with tryptophan. I have reference to the L-form of this substance, which is active in human metabolism, and which I am using clinically in a variety of combinations with B vitamins to good advantage. Tryptophan is an essential amino acid which may be derived from the metabolism of a variety of animal and vegetable proteins eaten by man. Allegedly, the metabolism of L-tryptophan yields the following (slide):



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Here we have serotonin (5-hydroxytryptamine) which chemically is closely related to 4-hydroxytryptamine. The quieting effects derived from serotonin which result seemingly from its stimulation of the parasympathetic-autonomic system have been extensively studied, and essentially consist clinically of lowered temperature, narrowed pupils, diminished pulse rate, decreased blood pressure, and subjectively less tension or anxiety. This is actually the opposite clinical picture to euphoria. However, when one turns to the other prominent metabolite of L-tryptophan, namely, niacin or nicotinic acid, entirely different inferences might be drawn (slide no. 5). A considerable literature exists to inform you that a deficiency of this substance or its precursor tryptophan as has occurred in large populations on this planet has been the basis for wide-spread pellagra, a condition characterized by mental changes, such as, anxiety, hallucinations, depression, and disorientation. In my clinical observations with niacin, either as it might be derived in exaggerated amounts from the L-tryptophan or where applied in its isolated state, a variety of results occur which suggest that many tissues are affected by the niacin; but often among these findings are those of improved energy coefficient and mood modified upwards to the level of almost mania in some. It is my impression that the niacin factor has a huge enterprise in the body. Whether its excitatory effect is accomplished through its known action in the Krebs cycle, or whether as an amine oxidase inhibitor, I am not certain. However, I am more impressed with its alleged role in the formation of diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN), which substances join the general metabolism to cellular energy. It is reported that niacin is involved in 35 important, separate metabolic steps. The above line of reasoning inevitably leads one to consider the probability that effects in or on enzyme systems will probably be found as the basis of the mushroom observations.

Besides the dosage being important in these various substances as to the effects produced from individual to individual, must we not also take into consideration the various possible pathways of such a substance as niacin which each human may have inherited or which exist in us as potentials to assert themselves under differing metabolic conditions? Is this a part of what is meant by the biochemical individual differences that are encountered in this class of research which involves nervous system and metabolism. My work with L-tryptophan has shown that many adjustments of nervous system functioning can be secured through its correct manipulation. I believe that somewhere in its metabolism there exists close relationships to the effects observed with whole mushrooms or isolated compounds secured from them. With specific reference to mushrooms, probably several chemical compounds, working alone or in combination, will be found to be psychoneurophysiologically effective in each significant mushroom, and not only those related to tryptophan as I have been suggesting. Useful explanations will be uncovered for such unusual phenomena which have been alleged or reported, and which I believe have occurred in situations where so-called "sacred" mushroom material has been ingested, such as extra-sensory perception, psi-activity and hallucinosis. To me, it is understandable how unsophisticated or primitive people came to label this material "sacred".

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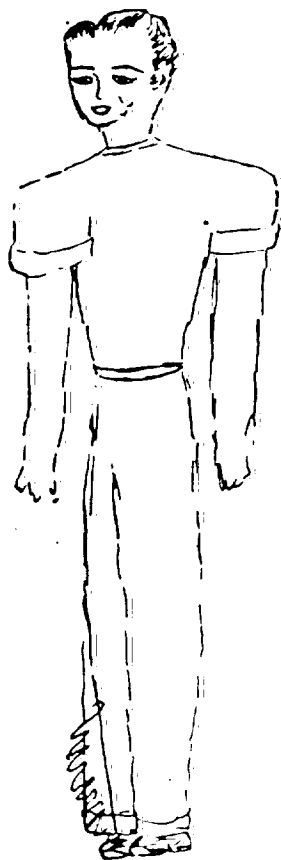
In closing, I should like to prognosticate that conceivably man's total maturity of neural functioning, including his mood, motivation, energy, even quality of thought, and some currently unsolved medical conditions, eventually may be significantly determined by manipulating combinations of food, and/or of its ingredients, and/or of other biological vegetations which are in the process of being explored. The term, psychodietetics, used recently at a nutrition symposium seems very apt. In that context, the quality and quantity of what one would write in a paper to be presented to a society might even depend considerably on whether he was ingesting candy-bars, pretzels, L-tryptophan or mushrooms while writing.

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SLIDES



No. 1



No. 2



Draw-A-Person

April 16, 1958  
(before treatment)

Draw-A-Person

June 3, 1958  
(after treatment)

Draw-A-Person

April 16, 1958  
(before treatment)

Draw-A-Person

June 3, 1958  
(after treatment)

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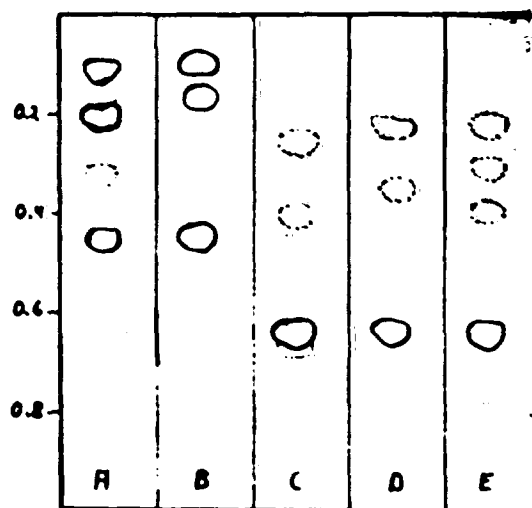


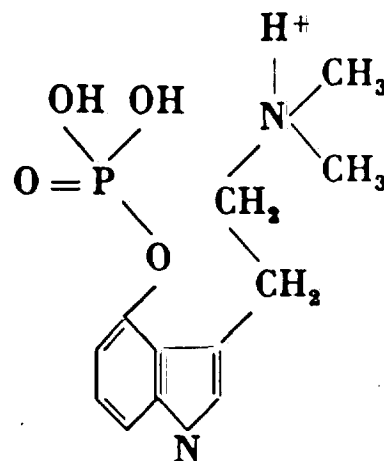
Fig. 1  
Paper Chromatograms of

- A, *P. venenosus*
- B, *P. sphrincrinus*
- C, *Psil. mexicana*
- D, *Psil. cubensis*
- E, *Psil. caerulescens*

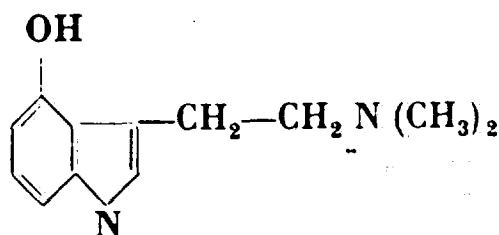
Solvents: Isopropanol - water 9:1

Detecting reagent: Ehrlich's reagent.

No.3



Psilocybin



4-Hydroxytryptamine

No.4

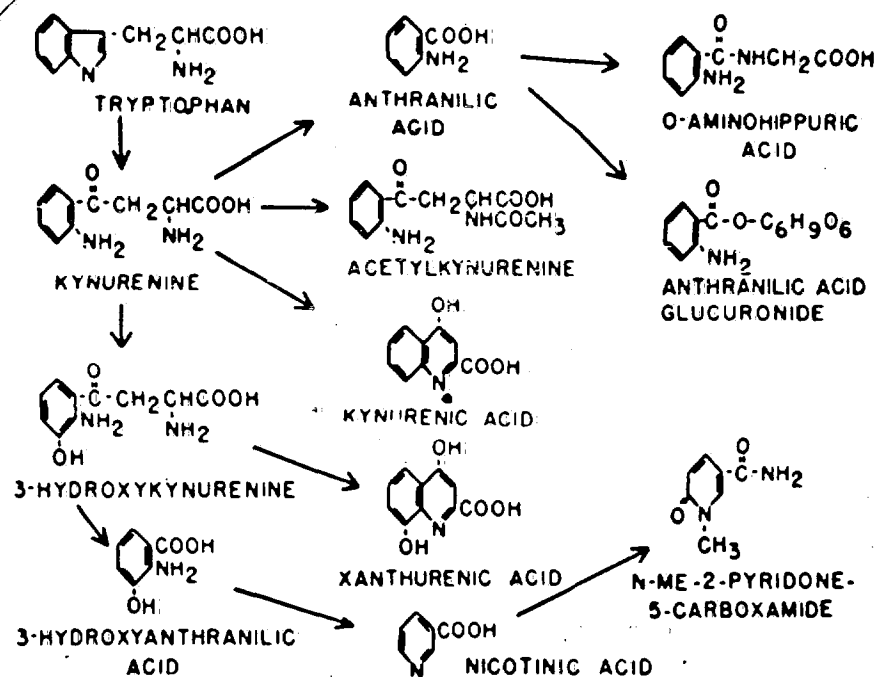


Fig. 1.—Abbreviated diagram of the pathway from tryptophan to niacin and its chief metabolite, N-methyl-2-pyridone-5-carboxamide, showing the interrelationships of the various metabolites. This figure has been published previously.<sup>40,41</sup>

6. Budget Plan:

TOBACCO INDUSTRY RESEARCH COMMITTEE

For First Year 350 FIFTH AVENUE NEW YORK 1, N. Y.

Dependable in 1955

Application For Research Grants

Other 10%

Date:

Total

June 15, 1955

7. Anticipated Duration of Project

1. Name of Investigator:

ARTHUR H. STEINHAUS

8. Technical Staff

2. Title:

Professor of Physiology

3. Institution

& Address: George Williams College

5315 South Drexel Avenue, Chicago 15, Illinois

4. Project or Subject:

Does tobacco smoke influence the production or action of sex hormone. To

9. Additional Remarks:

date we have shown that (1) tobacco smoke reduces spontaneous activity in the white rat, (2) this reduction is roughly proportional to the amount of nicotine in the tobacco, (3) in castrates there is no reduction attributable to smoking, (4) we have indication that with the injection of male hormone the effect observed in normal rats can be reproduced in castrates, (5) the estral cycle as observed by vaginal smear technique is not modified by two months of exposure to tobacco smoke. We would now like to do the following:

1. Repeat castrate study with graduated hormone additions.
2. Observe vaginal smears in spayed rats with graduated hormone additions.
3. Develop technique and observe effect of smoking on sex hormone excretion in man.
1. Double our capacity to study spontaneous activity of rats in fresh and smoke-laden air.
2. Compare our method of producing and administering cigarette smoke with that developed by others implied in Mr. Hockett's letter. Currently we use continuous suction which "smokes" one cigarette in 3-4 minutes into a closed box containing rats. This is repeated at hourly intervals for twelve hours per day.
3. Observe spontaneous activity in castrate males and females in fresh air and in smoke to determine a base line of activity and then observe increases due to the addition of small graduated amounts of testosterone or estrin. We would then determine what levels of hormone administration are offset by "smoking."
4. Determine minimal amounts of estrin necessary to activate the vaginal cycle in spayed rats and then determine if smoking is able to offset the effect of such minimal amounts.
5. Prepare for studies on man even while we are doing this animal work by perfecting ourselves in methods of assaying the urinary steroids in man so as to determine if smoking will produce quantitative or qualitative changes in them. Within the first year we would seek to perfect our methods and perhaps get some indications of the effect of smoking. In a second year we should begin to get more conclusive data on the effect of smoking on the steroid picture in man.

Signature

Director of Project

1003541277

**TORACCO**

For first year

## Salaries

## Expendable Supplies

### Permanent Equipment

Applic: Overhead ~~12-17-10~~ \*\*See below

Other 10%

Total

**# 10065**

**7. Anticipated Duration of Work:**

the same of kindness

At least two years will be needed to clarify the questions raised, provided some positive findings turn up. If the experimental plans here outlined are fruitless this should become obvious after one year.

8. Outlines are available

2. Life-

Title: A three room completely detached laboratory with thermostatic control of temperature (no provision for cooling air). Standard laboratory equipment.

3. **Staff:** Clayton F. Holoway, Assistant Professor, research chemist currently on summer assignment with Argonne Laboratory in steroid research.

Three graduate students -- two with one year of experience in the project.  
Arthur H. Steinhaus, Professor of Physiology -- Director of the project.

A. Project or subject: 1942-43

## 9. Additional Requirements

9. Additional Requirements: We have a Coleman photoelectric colorimeter. We may need a second one -- a spectrophotometer. This would be a requirement for the second half year. Also the spectrophotometer is a piece of equipment which can be repaired in the laboratory. (3) The spectrophotometer is a piece of equipment which is not satisfied by two months of exposure to the sun. We would like to do the following:

1. Hesperis matronalis with gaudy red flowers and leaves.

10. Additional Information (Including relation of work to other projects and other sources of supply):

[illegible]

It is conceivable that from a study such as this one some clues may emerge concerning

1. a mechanism for possible sex differences in susceptibility to tobacco smoke. John G. Reprints of a preliminary report of our work are attached.

[illegible]

2711 \*Salaries: 24 2711-01: 241 2711-02: 242 2711-03: 243 2711-04: 244 2711-05: 245 2711-06: 246 2711-07: 247 2711-08: 248 2711-09: 249 2711-10: 250 2711-11: 251 2711-12: 252 2711-13: 253 2711-14: 254 2711-15: 255 2711-16: 256 2711-17: 257 2711-18: 258 2711-19: 259 2711-20: 260 2711-21: 261 2711-22: 262 2711-23: 263 2711-24: 264 2711-25: 265 2711-26: 266 2711-27: 267 2711-28: 268 2711-29: 269 2711-30: 270 2711-31: 271 2711-32: 272 2711-33: 273 2711-34: 274 2711-35: 275 2711-36: 276 2711-37: 277 2711-38: 278 2711-39: 279 2711-40: 280 2711-41: 281 2711-42: 282 2711-43: 283 2711-44: 284 2711-45: 285 2711-46: 286 2711-47: 287 2711-48: 288 2711-49: 289 2711-50: 290 2711-51: 291 2711-52: 292 2711-53: 293 2711-54: 294 2711-55: 295 2711-56: 296 2711-57: 297 2711-58: 298 2711-59: 299 2711-60: 300 2711-61: 301 2711-62: 302 2711-63: 303 2711-64: 304 2711-65: 305 2711-66: 306 2711-67: 307 2711-68: 308 2711-69: 309 2711-70: 310 2711-71: 311 2711-72: 312 2711-73: 313 2711-74: 314 2711-75: 315 2711-76: 316 2711-77: 317 2711-78: 318 2711-79: 319 2711-80: 320 2711-81: 321 2711-82: 322 2711-83: 323 2711-84: 324 2711-85: 325 2711-86: 326 2711-87: 327 2711-88: 328 2711-89: 329 2711-90: 330 2711-91: 331 2711-92: 332 2711-93: 333 2711-94: 334 2711-95: 335 2711-96: 336 2711-97: 337 2711-98: 338 2711-99: 339 2711-100: 340 2711-101: 341 2711-102: 342 2711-103: 343 2711-104: 344 2711-105: 345 2711-106: 346 2711-107: 347 2711-108: 348 2711-109: 349 2711-110: 350 2711-111: 351 2711-112: 352 2711-113: 353 2711-114: 354 2711-115: 355 2711-116: 356 2711-117: 357 2711-118: 358 2711-119: 359 2711-120: 360 2711-121: 361 2711-122: 362 2711-123: 363 2711-124: 364 2711-125: 365 2711-126: 366 2711-127: 367 2711-128: 368 2711-129: 369 2711-130: 370 2711-131: 371 2711-132: 372 2711-133: 373 2711-134: 374 2711-135: 375 2711-136: 376 2711-137: 377 2711-138: 378 2711-139: 379 2711-140: 380 2711-141: 381 2711-142: 382 2711-143: 383 2711-144: 384 2711-145: 385 2711-146: 386 2711-147: 387 2711-148: 388 2711-149: 389 2711-150: 390 2711-151: 391 2711-152: 392 2711-153: 393 2711-154: 394 2711-155: 395 2711-156: 396 2711-157: 397 2711-158: 398 2711-159: 399 2711-160: 400 2711-161: 401 2711-162: 402 2711-163: 403 2711-164: 404 2711-165: 405 2711-166: 406 2711-167: 407 2711-168: 408 2711-169: 409 2711-170: 410 2711-171: 411 2711-172: 412 2711-173: 413 2711-174: 414 2711-175: 415 2711-176: 416 2711-177: 417 2711-178: 418 2711-179: 419 2711-180: 420 2711-181: 421 2711-182: 422 2711-183: 423 2711-184: 424 2711-185: 425 2711-186: 426 2711-187: 427 2711-188: 428 2711-189: 429 2711-190: 430 2711-191: 431 2711-192: 432 2711-193: 433 2711-194: 434 2711-195: 435 2711-196: 436 2711-197: 437 2711-198: 438 2711-199: 439 2711-200: 440 2711-201: 441 2711-202: 442 2711-203: 443 2711-204: 444 2711-205: 445 2711-206: 446 2711-207: 447 2711-208: 448 2711-209: 449 2711-210: 450 2711-211: 451 2711-212: 452 2711-213: 453 2711-214: 454 2711-215: 455 2711-216: 456 2711-217: 457 2711-218: 458 2711-219: 459 2711-220: 460 2711-221: 461 2711-222: 462 2711-223: 463 2711-224: 464 2711-225: 465 2711-226: 466 2711-227: 467 2711-228: 468 2711-229: 469 2711-230: 470 2711-231: 471 2711-232: 472 2711-233: 473 2711-234: 474 2711-235: 475 2711-236: 476 2711-237: 477 2711-238: 478 2711-239: 479 2711-240: 480 2711-241: 481 2711-242: 482 2711-243: 483 2711-244: 484 2711-245: 485 2711-246: 486 2711-247: 487 2711-248: 488 2711-249: 489 2711-250: 490 2711-251: 491 2711-252: 492 2711-253: 493 2711-254: 494 2711-255: 495 2711-256: 496 2711-257: 497 2711-258: 498 2711-259: 499 2711-260: 500 2711-261: 501 2711-262: 502 2711-263: 503 2711-264: 504 2711-265: 505 2711-266: 506 2711-267: 507 2711-268: 508 2711-269: 509 2711-270: 510 2711-271: 511 2711-272: 512 2711-273: 513 2711-274: 514 2711-275: 515 2711-276: 516 2711-277: 517 2711-278: 518 2711-279: 519 2711-280: 520 2711-281: 521 2711-282: 522 2711-283: 523 2711-284: 524 2711-285: 525 2711-286: 526 2711-287: 527 2711-288: 528 2711-289: 529 2711-290: 530 2711-291: 531 2711-292: 532 2711-293: 533 2711-294: 534 2711-295: 535 2711-296: 536 2711-297: 537 2711-298: 538 2711-299:

3. ~~Cost~~ One x-trained chemist 1/4 to 1/3 time ~~for 120 months and~~ in 120 months and in \$2,000

One graduate student - chemist - 1/2 time - 10 months - \$1,800

at \$21. One graduate student - histologist - 1/2 time for 10 months - \$1,800

One graduate student - technician-physiologist - 1/2 time

4. Derivation of Assets: Amounts of net worth for the 10 months is 1,800

5. Program for utilization of resources for the purpose of the project is as follows: \$7400

**\*\*Permanent Equipment**

10 exercise wheels @ \$85

A second smoke box and smoking machine as intimated in Mr. Hockett's letter (this is a wild guess)

0115

Signature /s/ Arthur H. Steinhaus

Director of Project: 731212.28

/s./ not distinguishable

Business Officer of the Institution and a

President

COPY

STEPHANO BROTHERS

Philadelphia 7, Pa.

March 26, 1959

Dr. Robert C. Hockett  
Tobacco Industry Research Committee  
150 E. 42nd Street  
New York 17, New York

Dear Dr. Hockett:

Was very pleased to receive your letter of March 19th with your comments on the results of the paramecium test on the five coded samples.

In reference to your suggestion for a titration to evaluate the relative carcinogenicity of the compounds, we have already done this using the activity of 3.4 Benzpyrene as the standard of comparison. We also have another evaluation which we call photodynamic toxicity. The results are as follows:

No.	Identity	Potency as Recorded for Mouse Skin	Photodynamic Toxicity %	Carcinogenic Activity based on 3.4 Benzpyrene
0	3:4 Benzpyrene	Potent	100	100%
1	2-Methyl-3,4-Benz-phenanthrene	Unknown	130	43
2	1,2,5,6-Dibenzanthracene	Weak	81	0
3	3-Methyl Cholanthrene	Potent	83	121
4	9-Methyl anthracene	Inactive or very low	79	0
5	3,4,9,10-Dibenzpyrene	Very potent	31	200

The reason I did not originally report the relative activity was that I thought that you just wanted to know if the paramecium test showed activity or not. Further, the scale is based on an assumed linear relationship which I do not know whether or not it is sound assumption. I was quite disturbed that I missed the activity of 1,2,5,6 Dibenzanthracene reporting it as zero when the mouse test shows a weak activity. Anyway, we know that it is quite less than 43% of the activity of 3.4 Benzpyrene.

In searching the literature I have found the following references on the activity of 1,2,5,6 Dibenzanthracene.

Wolmay Chemical Abstracts 1940 P. 4469

"Has a proliferative effect on paramecium at an optimum concentration of 1 in  $2 \times 10^5$ "

Further Mottram Cancer Research Vol. 1, P. 313 said

"Leads to production of abnormal forms of paramecia even in the dark"

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and further Mottram & Doniach, Nature 140 P. 933 said

"Does not affect the motility of paramecium in the dark but at a concentration of 1 in  $10^4$  it is rapidly lethal on irradiation with light of wave length of 350-410 m $\mu$  or with sunlight has similar activity on the infusorium coleps"

From the above two references giving concentrations of 1,2,5,6-Dibenzanthracene these reactions discussed occur at concentrations of 20-200 times stronger than those used in my tests.

I chose the level of concentration for the test of these unknowns on the basis of the level I used in calibrating the 3:4 benzpyrene curve for my experimental set-up and the curve gives the best response at a concentration of 1 in  $10^6$  to 1 in  $10^8$ . It would seem that this test of an unknown should be conducted as I dealt with it in the case of the five unknowns to separate the strong from the weak but when the first test indicates a weak or zero activity further tests should be run in the 1 part in  $10^3$  to 1 part in  $10^5$  level.

This should enable us to escape overlooking weak activity and evaluate same in terms of the activity of 3:4 Benzpyrene. On this basis I roughly estimate the activity of 1,2,5,6-Dibenzanthracene to lie between 0.5%-5.0% of the carcinogenic activity of 3:4 Benzpyrene.

In reference to further evaluation of the test, as I told you during our recent meeting I am very busy with work in the nature of cigar smoke so it would be very good if you could interest some academic institution to further explore the method; as to response of all known P.A.H.C. of carcinogenic nature; to the paramecium test.

Further it would be very good if the Scientific Advisory Board of the T.I.R.C. chose to have normal cigarette smoke as I define it in my paper checked against abnormal cigarette smoke on mouse skin tests being sure that the smoke used is normal or abnormal in the basis of the M.E.T. rise puff by puff. This would really answer the question under consideration on the basis of methods accepted today.

Further statistical studies of the population on samples properly chosen from the statistical viewpoint would confirm or deny my proposition that the average smoker does not exceed an M.E.T. rise of  $5.1^{\circ}\text{C}$ .

M.E.T. Rise Studies on persons afflicted with lung cancer would be of great interest.

These tests and others that the Scientific Advisory Board of the T.I.R.C. might think of, such as tissue culture test, would enable us to challenge on a firm basis the Wynder thesis using his own tools. With best regards, I am

Very sincerely yours,  
STEPHANO BROTHERS

/s/ C. S. Stephano

C. S. Stephano

CSS:vp

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RECEIVED

JUN 5 1971  
PHILIP MORRIS, INC.  
RESEARCH & DEVELOPMENT  
DEPT.

1003541281



CROSS REFERENCE SHEET

Name or Subject

Murray M. Streitfeld

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Regarding

Re Grant #82A

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---

SEE

Milton S. Saslow

---

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1003541282

TOBACCO INDUSTRY RESEARCH COMMITTEE  
350 FIFTH AVENUE NEW YORK 1, N. Y.

Salaries  
Expendable Supplies  
Application For Research Grant

Overhead

Other (Travel, Postage, etc.)  
Analyses, etc. Date: *October 6, 1954*

*Smoke tests w/ lot exp 33*  
*How much?*  
*How reacted?*  
*How at all - are 9 much ext.*  
*Caution of tobacco smoke*  
*The finished*  
*post* *Arrive*

4. Budget Plan:

a) Marion B. Sulzberger, M. D.

7. Name of Investigator: b) Will Cook Spain, M. D.

c) Rosa Lee Memir, A.B., M.D.

a) Professor and Chairman, Dept. of Dermatology & Syphilology, New York University Post-Graduate Medical School; Director, New York Skin and Cancer Unit.  
b) Professor of Clinical Medicine, Director of Allergy Section.  
c) Associate Professor of Pediatrics, New York University School of Medicine, New York City.

8. Institution: New York University - Bellevue Medical Center, 1300 First Avenue, New York 16, N. Y.

4. Project or Subject:

Investigation of the effects of tobacco on the human vascular system in living volunteers; and in particular of the possibility that certain tobacco effects are based on peculiar allergic susceptibility of specific individuals rather than upon obligatorily toxic products in tobacco smoke: origin, cigarette

5. Detailed Plan: Previous studies by the first named Applicant (see attached reprints) and by others have shown that extracts of cigarette and other tobacco are capable of producing urticarial reactions in a certain percentage of human beings -- and that the incidence of these reactions is higher in:  
I. Patients with thrombo-angitis obliterans;  
II. Patients with other cardiovascular diseases sometimes associated with smoking;

III. Heavy smokers.  
It has also been demonstrated that a significant drop in temperature of the digits during cigarette smoking is demonstrable in about 30% of subjects treated. (Reddish). This figure of approximately 30% corresponds to the approximately 30% of smokers found to react with urticarial reactions to skin tests with tobacco -- but it is not known whether it is the same 30% -- i.e. whether those subjects with significant falls in temperature are also those with the urticarial skin responses.

It is planned to carry out the following critical experiments to establish whether or not specific allergic sensitization plays a basic role in vascular reactions to tobacco smoking:

A) Infants and children will be skin tested with tobacco extracts from various brands of cigarettes, and from various types of uncured tobacco, to ascertain whether the incidence of positive reactions increases with age or is related to exposure to tobacco (families of smokers and non-smokers etc.)

B) Adolescents and young adults will be tested to ascertain whether there is a significant increase in incidence of skin reactions at the time of beginning smoking.

(Continued on attached page)

By *Marion B. Sulzberger*, Supervisor of Finance

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5. Continued.

C) Patients with various vascular and other diseases which have sometimes been regarded as being made worse by smoking will be skin tested to ascertain whether their skin reactions are in higher incidence or greater intensity than those of control subjects of equivalent age and exposure to tobacco.

D) Volunteers will have the effects of smoking upon the temperature of their ~~exp~~ extremities accurately measured by the most exact modern instruments and will also have skin tests with a ~~large~~ battery of tobacco extracts -- to see whether there is a positive correlation between reduction in peripheral temperature and reactions to skin tests.

E) All the skin test ~~re~~ reactions from the battery of tobacco extracts will be statistically analyzed, not only in regard to host facts (such as age, tobacco exposure, smoking allergic and hereditary constitution, disease, etc.), but also in regard to relative capacity of various brands and types of tobacco to produce sensitization of the vascular ~~&~~ tree ("index of sensitization", "relative sensitizing potential").

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## 6. Budget Plan:

TOBACCO INDUSTRY RESEARCH COMMITTEE  
350 FIFTH AVENUE NEW YORK 1, N. Y.

Salaries  
Expendable Supplies  
Applicable Permanent Equipment

\$ 10,000 per Annum

500 " "

1,500 " "

2,500 per Annum

500 " "

\$ 15,000

Other (Travel, Biostatistical analyses, etc.)

Date:

Total

a) Marion B. Sulzberger, M. D.

7. Anticipated Duration of Work: Will Cook, M. D.

a) Two years.

a) Professor and Chairman, Dept. of Dermatology &amp; Syphilology, New York University

8. Facilities and Staff Available: Medical School, University Hospital, New York Skin and Cancer Unit.

b) Professor of Clinical Medicine, Department of Surgery Section.

c) Complete facilities and patients and selected staff of the Departments specified above at the New York Skin and Cancer Unit, University Hospital, Bellevue Hospital, Gouverneur Hospital and other teaching services of New York University-Bellevue Medical Center.

New York 16, N. Y.

9. Additional Requirements:

Investigation of the effects of tobacco on the human circulatory system in living

The most modern studies on the effects of smoking upon the temperature

of the extremities have been carried out in this medical center by Walter Reddish,

Supply of pure tobacco of different types and origin, cigarette tobacco of different brands.

10. Additional Information (including relation of work to other projects and other sources of supply):

The most modern studies on the effects of smoking upon the temperature of the extremities have been carried out in this medical center by Walter Reddish, M. D. (Goldwater Memorial Hospital). Dr. Reddish's experience with apparatus and techniques, advice and guidance should prove invaluable in that part of the study relating to temperature effects.

Dr. Spain's experience and the facilities of his technical staff and laboratories (Miss Margaret Strauss) should prove invaluable in the preparation and control of the tobacco extracts and skin testing.

The experience and clinical material of Dr. Nemir and the Department of Pediatrics (Dr. Adolf DeSanctis) and the help of the Biostatistical Department (Dr. Donald Mainland) should also be of the very greatest value in the design and execution of the study and the evaluation of the results.

It is planned to carry out the following critical experiments to determine whether or not specific allergic sensitization plays a basic role in tobacco-induced tobacco sickness.

A) Infants and children will be skin tested with tobacco extracts from various brands of cigarettes and from various types of smoked tobacco, to determine whether the incidence of positive reactions increases with age or is related to exposure to tobacco (family of smokers and non-smokers etc.).

B) Adolescents and young adults will be skin tested with tobacco extracts to determine whether or not the incidence of positive reactions is related to exposure to tobacco (family of smokers and non-smokers etc.).

Signature

Director Marion B. Sulzberger

(Continued on attached page)

Budget Officer: Edward E. Smith, Supervisor of Finance

1003541285

Secretary  
Expendable Budget  
Application For Research Grant

Overhead  
Other

#33 R1,000 per annum

Date: Total

December 8, 1955

## 1. Name of Investigator: Work

- a) Marion B. Sulzberger, M.D.  
b) Will Cook Spain, M.D.

## 2. Title:

- c) Rosa Lee Nemir, M.D. and her staff and selected staff of the  
d) Vincent J. Fontana, M.D. in the Cancer Unit, New York University  
a) Professor & Chairman, Dept. of Dermatology & Syphilology, New York University,  
Post-Graduate School Medical, Director, N. Y. Skin and Cancer Unit; b) Prof. of  
Clinical Medicine, Director of Allergy Section; c) Prof. of Pediatrics  
d) Instructor in Clinical Pediatrics

New York University - Bellevue Medical Center  
550 First Avenue, New York 16, N.Y.

Investigation of the effects of tobacco on the human vascular system in living volunteers; and in particular of the possibility that certain tobacco effects are based on peculiar allergic susceptibility of specific individuals rather than upon obligatorily toxic products in tobacco smoke.

## 5. Detailed Plan of Procedure (Use reverse side if additional space is needed):

studies on the effects of smoking upon the temperature. Previous studies by the first-named Applicant (see attached reprints) and by others have shown that extracts of cigarette and other tobacco are capable of producing urticarial reactions in a certain percentage of human beings - and that the incidence of these reactions is higher in certain groups of patients. The first group (I) consists of patients with thrombo-angiitis obliterans; the second group (II) consists of patients with other cardiovascular diseases sometimes associated with smoking; the third group (III) consists of heavy smokers.

It has also been demonstrated that a significant drop in temperature of the digits during cigarette smoking is demonstrable in about 30% of the subjects treated. (Reddish) This figure of approximately 30% corresponds to the approximately 30% of smokers found to react with urticarial reactions to skin tests with tobacco-but it is not known whether it is the same 30%-i.e. whether those subjects with significant falls in temperature are also those with the urticarial skin responses.

It is planned to carry out the following critical experiments to establish whether or not specific allergic sensitization plays a basic role in vascular reactions to tobacco smoking:

- a) Infants and children will be skin tested with tobacco extracts from various brands of cigarettes, and from various types of uncured tobacco, to ascertain whether the incidence of positive reactions increases with age or is related to exposure to tobacco (families of smokers and non-smokers, etc.)

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(Continued on page 2)

Secretary of the Institution

# 6. Budget Plan:

Salaries	\$10,000 per annum
Expendable Supplies	500 "
Applicable Permanent Equipment	1,500 "
Overhead	2,500 "
Other	500 "
Total	\$15,000

December 8, 1955

# 7. Anticipated Duration of Work:

Dr. Marion B. Sulzberger, M.D.  
Two Years.

# 8. Facilities and Staff Available:

Complete facilities and patients and selected staff of the Departments specified above at the New York Skin and Cancer Unit, University Hospital, Bellevue Hospital, Gouverneur Hospital, and other teaching services of New York University-Bellevue Medical Center. Dr. Spain, M.D., Prof. of Clinical Medicine, Director of Allergy Service, Prof. of Pediatrics, Instructor in Clinical Pediatrics.

University - Bellevue Medical Center  
350 First Avenue, New York 16, N.Y.

# 9. Additional Requirements:

Investigation of the effects of tobacco on the human vascular system. Supply of pure tobacco of different types and origin, cigarette tobacco of different brands, peculiar allergic susceptibility of specific individuals rather than upon obligatorily toxic products in tobacco smoke.

# 10. Additional Information (including relation of work to other projects and other sources of supply):

The most modern studies on the effects of smoking upon the temperature of the extremities have been carried out in this Medical Center by Walter Reddish, M.D. (Goldwater Memorial Hospital). Dr. Reddish's experience with apparatus and techniques, advice and guidance should prove invaluable in that part of the study relating to temperature effects. Dr. Spain's experience and the facilities of his staff (Dr. Vincent J. Fontana) and laboratories (Miss Margaret Strauss) should prove invaluable in the preparation and control of the tobacco extracts and skin testing.

The experience and clinical material of Dr. Nemir and the Department of the Allergy Pediatrics (Dr. Adolf G. DeSanctis) and the help of the Biostatistical Department (Dr. Donald Mainland) should also be of the very greatest value in the design and execution of the study and the evaluation of the results. It is not known whether it is the same or not, i.e. whether those subjects with significant falls in temperature are also those with the vertical skin responses.

It is planned to carry out the following critical experiments to establish whether or not specific allergic sensitization plays a basic role in vascular reactions to tobacco smoking:

a) Infants and children will be skin tested with tobacco extracts from various brands of cigarettes, and from various sources of tobacco. To ascertain whether the incidence of positive reactions is greater in infants and children who are tobacco smokers (families of smokers and non-smokers, etc.)

(Continued on page 2)

Business Officer of the Institution

**CONFIDENTIAL**

TIRC Grant #33

Progress Report #1

Dr. Marion B. Sulzberger  
New York University-Bellevue Medical Center

December 15, 1955

"Investigation of the Effects of Tobacco on the Human Vascular System in Living Volunteers; and in Particular of the Possibility that Certain Tobacco Effects are Based on Peculiar Allergic Susceptibility of Specific Individuals Rather than upon Obligatorily Toxic Products in Tobacco Smoke"

This is a report of the first 500 volunteers tested with tobacco extracts at the Judson Health Center in New York City. The individuals were mainly adults in the lower income group.

The volunteers were skin tested by the intracutaneous method with 1/20cc of five different types of tobacco extracts. The site of testing was observed for at least 20 minutes to note result of test. Only the marked reactions were considered positive for this study. Permanent tracings were obtained on all marked positive reactions and normal saline was used as a control.

Before skin testing the individual, a thorough personal and familial history of allergy was obtained in conjunction with specific questioning about peripheral vascular symptoms and pertinent smoking habits.

The tobacco extracts were prepared by Miss Margaret Ballard Strauss, Director of the Allergy Laboratory at University Hospital. The usual methods of extraction were used\*. The following tobacco extracts were used for testing in 1000 unit phosphotungstic acid precipitable Nitrogen.

1. Burley
2. Virginia
3. Turkish
4. Mixed
5. Mixed (defatted)

The mixed tobacco extracts consisted of a mixture of equal amounts of popular brands of cigarettes - (Lucky Strike, Old Gold, Chesterfield, Camels, Pall Mall, Philip Morris).

One of the mixtures was made in the usual way by defatting with an organic solvent for the removal of fats and various organic components generally soluble in toluene or other similar fat solvents. After the tobacco was thus treated, it was then extracted with an aqueous extracting fluid. The non-defatted mixture was extracted with aqueous solution without previous treatment with organic solvent.

Dr. Walter Reddish and his staff at the Goldwater Memorial Hospital have studied volunteers that were skin tested in order to uncover any possible relationship between the skin test reactions and peripheral vascular disease. He has undertaken the following procedures:

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1. Ballistocardiograms
2. Electrocardiograms
3. Peripheral blood flow studies
4. Peripheral temperature changes

Recordings have been made before and after smoking special cigarettes composed of a mixture of the same brands used in making the tobacco extracts.

Unfortunately, these results have not been completely assembled and are not reportable at this time.

Respectfully submitted,

s/ Vincent J. Fontana, M.D.  
for Marion B. Sulzberger, M.D.

1003541289



# RESULTS

TABLE I

Individuals with <u>positive</u> skin tests*	79 (15.8%)
Individuals with <u>negative</u> skin tests	421 (84.2%)

\* Marked positive skin reactions to one or more tobacco extracts.

TABLE II

Which particular tobacco extracts gave the most, and the least, number of positive reactions?

## POSITIVE REACTIONS TO THE SPECIFIC TOBACCO EXTRACT

Burley	40 (50%)
Turkish	37 (46.8%)
Virginia	45 (57%)
Mixed	47 (59%)
Mixed (defatted)	30 (39%)

TABLE III

Do people with positive skin tests to tobacco have a tendency to more peripheral vascular symptoms?

	total	vascular symptoms
POSITIVE skin test individuals	79	18 (22%)
NEGATIVE skin test individuals	421	47 (11%)

TABLE IV

Do people who react positive by skin tests to tobacco have more tobacco symptoms than those that have negative skin tests?

	total	tobacco symptoms
POSITIVE skin test individuals	79	14 (16%)
NEGATIVE skin test individuals	421	17 (4%)

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TABLE V

Do people with familial and personal history of allergy give more positive skin reactions to tobacco:

	total	personal allergy	familial allergy
POSITIVE skin test individuals	79	25 (31%)	19 (22%)
NEGATIVE skin test individuals	421	54 (12.8%)	57 (13.5%)

TABLE VI

Do smokers with positive skin tests have more tobacco symptoms than smokers with negative skin tests?

Smokers	total	tobacco symptoms
POSITIVE skin tests	52	14 (26%)
NEGATIVE skin tests	259	17 (6.5%)

TABLE VII

Do smokers who have positive skin tests have more vascular symptoms than smokers with negative skin tests?

Smokers	total	Vascular symptoms
Positive skin tests*	52	16 (30.7%)
Negative skin tests**	259	32 (12%)

TABLE VIII

Do non-smokers who have positive skin tests to tobacco have more vascular symptoms than non-smokers with negative skin tests?

Non-smokers	total	Vascular symptoms
Positive skin test*	27	2 (7.4%)
Negative skin test**	162	15 (9.2%)

\* Individuals with positive skin tests.

\*\* Individuals with negative skin tests.

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## CRITERIA USED IN TOBACCO STUDY

### SKIN TESTS:

Only the marked positive skin tests (with pseudopods) were considered in the final count of positive reactions.

### VASCULAR SYMPTOMS:

Patient was considered to have positive vascular symptoms if three or more of the following signs and symptoms were present.

1. Fainting spells
2. Tingling of the extremities
3. Cold extremities
4. Numbness
5. Muscular aches and pains

### TOBACCO SYMPTOMS:

Patient was considered to have symptoms related to tobacco if two or more of the following reactions were experienced when smoking.

1. Coughing
2. Sweating
3. Sneezing
4. Headaches
5. Dizziness
6. Cold, numbness and tingling of the extremities

### PERSONAL ALLERGY:

Positive if the patient has experienced any of the following.

Hay fever  
Asthma  
Food Allergy

Drug sensitivity  
Atopic dermatitis

### FAMILIAL HISTORY:

Positive if any member of the patient's immediate family had the following.

Hay fever  
Asthma  
Food allergy

Drug sensitivity  
Atopic dermatitis

## IMPRESSIONS

1. 15.8% of the 500 individuals tested showed positive skin tests to one or more of the tobacco extracts. (This figure is in general agreement with those published for the incidence of allergy in the general population.)
2. There were more reactions to the mixed brands of tobacco extract non-defatted, and fewer reactions to the defatted mixed extract. The defatting process would remove an appreciable quantity of esters, aldehydes, and other organic compounds - ordinarily thought to be volatilized in the smoke of the cigarette. This would tend to indicate that a large number of individuals reacted to this volatile aromatic fraction alone.

1003541292

3. It would appear that vascular symptoms were reported twice as often in volunteers with positive skin tests than in those with negative skin tests.
4. Four times more individuals complained of tobacco symptoms if the skin tests were positive than if the skin tests were negative.
5. Individuals with positive skin tests to tobacco related twice as much evidence of personal and familial allergy than the negative skin test individuals.
6. Smokers with positive skin tests to tobacco are more likely to develop (4X more) tobacco symptoms than those with negative skin tests.
7. Smokers with positive skin tests had 2 1/2 times more vascular symptoms than smokers with negative skin tests.
8. In non-smokers the incidence of vascular symptoms was about the same whether the skin test was positive or negative.

s/ V. J. Fontana, M.D.

1003541293

TOBACCO INDUSTRY RESEARCH COMMITTEE  
150 EAST FORTY SECOND STREET NEW YORK 17, N. Y.

#160

Application For Research Grant

(Cf. #33 activated 2/1/55  
and renewed 2/1/56)

1. Name of Investigator: a) Marion B. Sulzberger, M.D.  
b) Walter Redisch, M.D.  
c) Vincent J. Fontana, M.D.  
d) Kurt De Crinis, M.D.
2. Title: a) Professor and Chairman, Dept. of Dermatology & Syphilology, N.Y. University  
Post-Graduate Medical School; Director, N.Y. Skin & Cancer.  
b) Associate Professor Clinical Medicine, N.Y. U. College of Medicine
3. Institution c) Assistant Clinical Professor Pediatrics, Post-Graduate Medical School  
& Address: d) Research Fellow  
New York University-Bellvue Medical Center  
550 First venue, New York 16, New York
4. Project or Subject:  
Investigation of the effects of tobacco on the human vascular system in healthy volunteers as well as in patients with occlusive vascular disease; particular attention to be directed at the possibility that certain tobacco effects are based on peculiar allergic susceptibility of specific individuals rather than upon obligatorily toxic products in tobacco smoke. Also, the possible influence of habitual smoking upon vascular responses is to be ascertained.
5. Detailed Plan of Procedure (Use reverse side if additional space is needed):

Previous studies by the first-named Applicant (see attached reprints) and by others have shown that extracts of cigarette and other tobacco are capable of producing urticarial reactions in a certain percentage of human beings - and that the incidence of these reactions is higher in:

1. Patients with thrombo-angitis obliterans.
2. Patients with other cardiovascular diseases sometimes associated with smoking.
3. Heavy smokers.

It has also been demonstrated that a significant drop in temperature of the digits during cigarette smoking is demonstrable in about 30% of subjects treated. (Redisch). This figure of approximately 30% corresponds to the approximately 30% of smokers found to react with urticarial reactions to skin tests with tobacco, but it is not known whether it is the same 30% -- i.e. whether those subjects with significant falls in temperature are also those with the urticarial skin responses.

1. The above investigators have found that 15.8% of the 500 individuals skin tested showed positive reactions to one or more of the tobacco extracts. (This figure is in agreement with those published - incidence of allergy in pop.)
2. There were more reactions to the mixed brands of tobacco extract which was not defatted, and fewer reactions to the defatted mixed extract.

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The defatting process would remove an appreciable quantity of esters, aldehydes, and other organic compounds--ordinarily thought to be volatilized in the smoke of the cigarette. This finding suggests that a large number of individuals reacted to this volatile aromatic fraction alone. In the group of positive cases tested, the individuals reacted to from one to five of the extracts. This would indicate that a person positive to tobacco skin test might prove sensitive to only one specific type of tobacco, and not to any other type.

3. Vascular symptoms were reported twice as often in volunteers with positive skin tests to tobacco than in those with negative skin tests.
4. Four times more individuals complained of tobacco symptoms when the tobacco skin tests were positive than when the skin tests were negative.
5. Individuals with positive skin tests to tobacco related twice as much evidence of personal and familial allergy than the negative skin test individuals.
6. Smokers with positive skin tests to tobacco presented four times more tobacco symptoms than those with negative skin tests.
7. Smokers with positive skin tests had  $2\frac{1}{2}$  times more vascular symptoms than smokers with negative skin tests.
8. In non-smokers the incidence of vascular symptoms was about the same whether the skin test was positive or negative.

It is planned to carry out the following experiments to establish whether or not specific allergic sensitization plays a basic role in vascular reactions to tobacco smoking:

- A) Patients with various vascular and other diseases which have sometimes been regarded as being made worse by smoking will be skin tested to ascertain whether their skin reactions are in higher incidence or greater intensity than those of control subjects of equivalent age and exposure to tobacco.
- B) Vascular responses are to be tested by automatic recording of surface temperature and large limb venous occlusion plethysmography, in a constant temperature-humidity room under environmental conditions  $\times$  set at 20° and 25° C temp. and with 55% humidity. Four groups will be studied:
  - (1) healthy volunteers, smokers; (2) healthy volunteers, non-smokers;
  - (3) patients with occlusive vascular disease, non-smokers. (4) patients with occlusive vascular disease - smokers. ECGs and BCGs before and after smoking will be done in all patients.
- C) The results will be correlated with the results of skin testing and clinical symptomatology.
- D) All the skin test reactions from the battery of tobacco extracts will be statistically analyzed, not only in regard to host factors (such as age, tobacco exposure, smoking, allergic and hereditary constitution, disease, etc.), but also in regard to relative capacity of various brands and types of tobacco to produce sensitization of the vascular tree ("index of sensitization", "relative sensitizing potential").

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6. Budget Plan:

Salaries	\$10,000 per annum
Expendable Supplies	500 " "
Permanent Equipment	1,500
Overhead (20%)	2,500 per annum
Other	500 " "
Total	\$ 15,000

7. Anticipated Duration of Work:

Two years

8. Facilities and Staff Available:

Complete facilities and patients and selected staff of the Departments specified above at the New York Skin and Cancer Unit, University Hospital, Bellevue Hospital, Gouverneur Hospital and other teaching services of New York University-Bellevue Medical Center. Completely equipped circulation laboratory of the New York University Research Service, Goldwater Memorial Hospital with staff of 4 physicians and 2 technicians.

9. Additional Requirements:

Supply of pure tobacco of different types and origin, cigarette tobacco of different brands.

10. Additional Information (Including relation of work to other projects and other sources of supply):

Studies of vascular responses have been carried out in the Medical Center by Walter Redisch, M.D. (N.Y.U. Research Service, Goldwater Memorial Hospital, under the direction of Dr. J. M. Steele) for many years. Dr. Redisch's experience, advice and guidance will be utilized widely in the Study relating to vascular responses. Dr. Vincent J. Fontana's experience and the facilities at the Allergy Laboratory under the direction of Miss Margaret Strauss should prove invaluable in the preparation and control of the tobacco extracts and skin testing.

Signature /s/ Marion B. Sulzberger  
Director of Project

Peter Acokellian (?)  
Business Officer of the Institution  
Business Manager

1003541296

TOBACCO INDUSTRY RESEARCH COMMITTEE  
150 EAST FORTY SECOND STREET NEW YORK 17, N. Y.

Application For Research Grant

#213  
(Cf. #3381 - Activated  
2/1/55  
Renewed - 2/1/56  
and #160 - Activated 8/1/57  
8/1/57

Date: September 10, 1958

1. Name of Investigator:
  - a) Marion B. Sulzberger, M.D.
  - b) Walter Hedisch, M.D.
  - c) Kurt DeCrisis, M.D.
  - d) Vincent Fontana, M.D.
2. Title:
  - a) Professor and Chairman, Dept. of Dermatology & Syphilology, New York Univ. Bellevue Med. Center, Post-Graduate Medical School
3. Institution:
  - b) Associate Professor Clinical Medicine, NYU College of Medicine.
  - c) Clinical Assistant
- & Address:

New York University - Bellevue Medical Center  
550 First Avenue, New York 16, New York
4. Project or Subject:

Investigation of the effects of tobacco on the human vascular system, based on the fact that certain tobacco effects are due to allergic susceptibility of specific individuals rather than to obligatorily toxic products in tobacco smoke. And that patients with occlusive vascular diseases respond differently than healthy smokers.

5. Detailed Plan of Procedure (Use reverse side if additional space is needed):

In the past two years our studies have shown that in about 30% of healthy subjects plethysmographically measured blood flow to the lower extremities decreased significantly (2 ml./100 cc/min. or more) for a transient period of time. 90% of these subjects who were negative on skin testing with tobacco extracts had no change in blood flow.

Thirty patients with clinically documented non-gangrenous obliterative arterio-sclerosis of the lower extremities have been tested so far. Thirteen of these had a significant (2 ml./100cc./min. or more) increase in plethysmographically measured blood flow to the lower extremities in response to tobacco smoking. The remaining 17 patients showed no change; none had a significant decrease.

These most puzzling findings urgently call for an extension of the study:

- 1) Further confirmation by increasing the number of test subjects in this group.
- 2) Testing of at least three more groups with other types of occlusive arterial disease, namely, patients with TAO\* patients with occlusive disease of small blood vessels and patients with "arteriospastic" disorders (Raynaud's reflex dystrophy) Vascular responses tested as before in association with intradermal testing with tobacco extracts.

\* TAO - Thrombo-angitis Obliterans



While no explanation can be offered at present for the surprising fact that some patients with OAS\*\* responded to tobacco smoking with vasodilation, it appears likely that this bears some relationship to altered vasomotor responses. Alterations of vasomotor responses in patients with various neurologic lesions have been under investigation by our group for the past several years. Studies of the basic mechanisms responsible for the constriction and dilation of blood vessels in man are in progress.

It is planned to carry out the following experiments to establish whether or not specific allergic sensitization plays a basic role in vascular reactions to tobacco smoking.

(A) Patients with various vascular and other diseases which have sometimes been regarded as being made worse by smoking will be skin-tested to ascertain whether their skin reactions are in higher incidence or greater intensity than those of control subjects of equivalent age and exposure to tobacco.

(B) Vascular responses are to be tested by automatic recording of surface temperature and venous occlusion large limb plethysmography in a constant temperature humidity room, under environmental conditions set at 20°C temperature with 55% humidity.

Four groups will be studied:

1. Healthy volunteers - smokers.
2. Healthy volunteers - non-smokers.
3. Patients with occlusive vascular disease - smokers
4. Patients with occlusive vascular disease - non-smokers.

ECG before and after smoking will be done on all patients.

(C) The results will be correlated with the results of skin-testing and clinical symptomatology.

(D) All the skin test reactions from the battery of tobacco extracts will be statistically analyzed, not only in regard to host factors (such as age, tobacco exposure, smoking, allergic and hereditary constitution, disease, etc.), but also in regard to relative capacity of various brands and types of tobacco to produce sensitization of the vascular tree ("index of sensitization", "relative sensitization potential").

\*\* OAS - Obliterative Arterio-Sclerosis

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6. Budget Plan:

Salaries	10,000	per annum
Expendable Supplies	500	" "
Permanent Equipment	2,000	" "
Overhead	2,000	" "
Other	500	" "
Total	15,000	

7. Anticipated Duration of Work: **Two years**

8. Facilities and Staff Available:

**Complete facilities and patients and selected staff of the Departments specified above at the New York Skin and Cancer Unit, University Hospital, Bellevue Hospital, Gouverneur Hospital and other teaching services of New York University-Bellevue Medical Center.**

9. Additional Requirements:

**Supply of pure tobacco of different types and origin, cigarette tobacco of different brands.**

10. Additional Information (Including relation of work to other projects and other sources of supply):

**All workers concerned are intimately familiar with the essential problems, having been engaged in this study for at least 2 years.**

Signature /s/ Marion E. Sulsberger  
Director of Project

/s/ Edward F. Smith  
Business Officer of the Institution

1003541299

Research Service  
Third New York University Medical Division  
The Goldwater Memorial Hospital  
Welfare Island, New York 17, N.Y.

February 20, 1959

Dr. Robert C. Hockett  
Associate Scientific Director  
Tobacco Industry Research Company  
150 East 42nd Street  
New York, N.Y.

Dear Doctor Hockett:

Thank you for the copy of your letter of February 4th to Dr. Sulzberger. I am glad to inform you that two pieces of the work are in the state of being published.

A paper, prepared by Dr. Sulzberger and Dr. Fontana, with our cooperation, has been accepted for publication in the Journal of Allergy.

A second paper, prepared by Dr. de Crinis, myself and Dr. Steele, has still to go through a last review by Dr. Steele. However, since it may be anticipated that further final changes by Dr. Steele will be minor, if any, (we have discussed the paper thoroughly), I am sending you a copy of the draft as it now stands.

In addition, I wish to inform you that we have, meanwhile, tested 45 patients with obliterative arteriosclerosis. Of these, 16 had a significant (more than 2 ml/100 tissue/min) increase in blood flow; 2 had a significant decrease; and 27 showed no significant change. No other form of obliterative arterial disease has been tested so far; thromboangitis obliterans will be next.

Of course, the recording of arterial pressure has been routine in all our experiments.

As far as the comparison between the effects of tobacco extract and the effects of smoke concentrate are concerned, both groups, Dr. Sulzberger and Dr. Fontana, as well as our group, are very ready, in fact anxious, to do this piece of investigation; as a matter of fact, we all had been talking about the possibility that this may well clear up the points of discrepancy between the allergic and the vascular responses.

The paragraph concerning financial details will be answered in a separate letter. Dr. Sulzberger, Dr. Fontana and I will discuss the details and report to you within a week or ten days.

Very sincerely,

/s/ Walter Redisch, M.D.

WR

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CONFIDENTIAL

TIRC Grants #33, -R1, 160, 213

Dr. Marion B. Sulzberger (Redisch)  
New York University-Bellevue Medical Center

Progress Report #2  
February, 1959

"Investigation of the Effects of Tobacco on the Human Vascular System in Healthy Volunteers as well as in Patients with Occlusive Vascular Disease; Particular Attention to be Directed at the Possibility that Certain Tobacco Effects are Based on Peculiar Allergic Susceptibility of Specific Individuals Rather than Upon Obligatorily Toxic Products in Tobacco Smoke. Also, the Possible Influence of Habitual Smoking Upon Vascular Responses is to be Ascertained."

### Introduction

There has been considerable interest in vasomotor responses to nicotine and to the smoking (or chewing) of tobacco for many years. There is almost complete agreement that smoking usually produces transient adrenergic stimulation via the sympathetic nervous system followed by depression of sympathetic and parasympathetic ganglia. Vasoconstrictive effect to tobacco smoking could not be elicited in sympathectomized limbs by the technique of toe plethysmography, which records skin flow only.

It has been fairly well established over the years that in a certain percentage of subjects, smoking of tobacco causes vasoconstriction of peripheral vessels as indicated by decrease in surface temperature and also by plethysmographically measured blood flow. The mechanism involved in this response has been the subject of some controversy. Mulinos and Shulman found that deep breathing per se may cause reflex vasoconstriction (and, consequently, diminution of peripheral blood flow measured plethysmographically) in fingers, hand and forearm. Bolton et al found similar changes. In contrast, Evans and Stewart believe that vasoconstrictor effect of smoking is neither due to nicotine nor to deep inhalation but rather to "irritation" by smoke per se. These authors based their opinion on the observation that smoke not containing any nicotine produced the same effect on peripheral circulation as did tobacco smoke, and that drawing on an unlighted cigarette produced no vasomotor effect. Others support the notion that nicotine is the effective agent and that smoking is merely a way of administering nicotine. Roth et al found that changes in peripheral vascular circulation produced by denicotinized and corn silk cigarettes were negligible.

Likewise, discrepant views concerning the vascular beds involved in constrictor responses to tobacco smoking have been expressed in the literature. Friedlander and associates found that following tobacco smoking the greatest decrease in blood flow occurred in the skin. This opinion is shared by other investigators. Abramson and co-workers believe that vasoconstriction following tobacco smoking is limited to the skin and that muscle flow is not affected at all. On the other hand, Fletcher found plethysmographic evidence of blood flow diminution of as much as 40%, without changes in surface temperature using an air transmission finger plethysmograph. He believes that skin temperature is not a reliable index of peripheral vascular response.

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Allergic-hyperergic mechanisms are also thought to be involved in the bodily response to nicotine, and the skin reaction to tobacco is believed by some to be a specific allergic response rather than an "irritative" reaction. Sulzberger showed that of 400 normal smokers, 32% reacted with a positive skin test to tobacco extracts and to extracts of timothy or horse dander; of these 32%, 9% reacted to tobacco only. Green reported that 13 of 100 normal smokers reacted positively to tobacco extracts. Romanoff and Rubin found 16 positive skin reactions to tobacco among 68 normal smokers. Trasoff reported 17% positive skin reactors in 40 normal smokers. The number of positive skin reactions to tobacco is obviously dependent upon the type of subjects used. It increases considerably when one is dealing with patients with thromboangitis obliterans. Cooke reported 78% positive skin reactions to 5 tobacco extracts and extracts of ragweed, timothy and horse dander in 140 patients with Buerger's Disease, compared with 9% positive skin reactions to tobacco in 400 unselected normal smokers. Forty-four of 95 patients with thromboangitis obliterans also demonstrated antibodies to tobacco on passive transfer. Sulzberger, Romanoff and Rubin reported similar findings.

Because of these observations it seemed of interest to correlate responses with sensitivity responses in the skin. During the study it became clear that in order to arrive at a definitive conclusion, different age groups, smokers and non-smokers, would have to be included and that comparison of patients with vascular disease with "healthy" subjects was necessary.

This first report deals merely with the findings in a group of 80 healthy smokers, none more than 50 years of age.

#### Methods and Material

Measurements of peripheral blood flow, recordings of surface temperature, heart rate and blood pressure were done in all subjects before and after smoking. Eighty healthy subjects were tested (27 females and 53 males), their ages ranging from 18 to 50 years with a mean of 36. Blood flow to the leg and foot was measured by means of a large limb venous occlusion plethysmograph, using an air transmission system connected to a strain gauge which permitted expression of very small pressure changes in terms of volume changes. Sudden venous occlusion was accomplished by a stopcock system, connected to a large pressure reservoir. Surface temperature was recorded continuously on a six-channel Leeds and Northrup Speedomax. All tests were performed in the Constant Temperature Room at 28°C and 55% humidity. Subjects were considered "adapted to the environment" when the toe temperature had remained constant for at least a  $\frac{1}{2}$  hour. All subjects were obliged to refrain from smoking and eating at least one hour before the test. Three plethysmographic base line tracings were obtained at five minute intervals after adaptation, then one test cigarette (containing a well equilibrated mixture of Burley, Virginia and Turkish tobaccos and mixed fatted and defatted tobacco) was smoked in six minutes. The amount of smoking of the single cigarette used here as stimulus was considered sufficient since it has been shown that one cigarette produced as much change as can be elicited in a given individual; a second cigarette does not alter the response. Subjects were encouraged to smoke in their accustomed manner. Three to four minutes after smoking was finished, another three tracings at five minute intervals were taken. Since electrocardiography and ballistocardiography have been widely used in assessing the effects of smoking on the coronary circulation, it was decided to

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add these methods to the measurement of peripheral blood flow. Electrocardiograms were taken, including standard leads, unipolar leads and V1, V3, V5; and ballistocardiography was performed simultaneously (D-V-A ballistocardiograph model A2-Arbeit)\*. Blood pressure and pulse rate were recorded before and after smoking. The following arbitrary criteria for significance of changes were accepted on the basis of previous experience:

1. Change of blood flow of at least 2 ml per minute per 100 ml tissue.
2. Change in surface temperature of at least 2°C.
3. Depressions of S-T and flattening of T waves ( $< 2$  mm) in the electrocardiogram.
4. Ballistocardiographic tracings were interpreted empirically, in a qualitative way on the basis of the following criteria:
  - Grade 0: Normal tracing; all IJK complexes normal in configuration and identical.
  - Grade I: Normal tracing; minor variations in IJK complexes which are still normal.
  - Grade II: Probably abnormal; significant variation in individual complexes, especially in the IJ wave.
  - Grade III: Abnormal; marked abnormalities in the individual complexes, some of which are still identifiable.
  - Grade IV: Markedly abnormal; the tracing is chaotic and there are no IJK complexes identifiable, as such.
5. Changes of arterial pressure of at least 20/20 mm Hg.
6. Change in heart rate of at least 15 beats/min.

The 80 subjects reported were also tested with intradermal injections of the (various) tobacco extracts (Burley, Virginia, Turkish, mixed fatted and defatted tobaccos) on the lateral aspects of the arm and reactions read after 10 minutes.\*\* The preparation of the tobacco extract was made according to the procedures which have been chosen by the Allergy Laboratory of the University Hospital of N.Y.U. from raw, untreated, sun-dried tobacco. Immediate wheal type reactions exceeding 10 millimeters were considered to be positive, and reactions subdivided into moderate and marked positive reactions (slightly positive reactions were not taken into consideration). Correlation of changes in blood flow with skin sensitivity was made subsequent to the independent observation.

## Results

Of the total 80 healthy subjects, 38 (47.5%) revealed changes in at least one of the above circulatory measurements after tobacco smoking, while 42 subjects (52.5%) showed no response. Only 10 subjects showed changes in the ballistocardiogram and only 3 in the EKG. We do not feel that any conclusions would be warranted from these figures.

\* - We wish to express our sincere thanks to Dr. Sidney Arbeit for supplying us with his D-V-A ballistocardiograph for this work and aiding us with his great experience in the interpretation of the ballistocardiograms.

\*\* - This part of the work was done by Drs. Sulzberger, Fontana and associates, who reported their results in detail at the 14th Annual Meeting of the American Academy of Allergy in Philadelphia, Pa., February 3, 1958.

subjects

Twenty-eight (35%) showed a significant decrease in the plethysmographically measured blood flow. Fifty-two (65%) showed no change, none showed an increase. We therefore concentrated on the evaluation of changes in plethysmographically measured extremity blood flow, in this first group of subjects.

Of the 80 subjects, 32 (40%) showed a positive skin reaction to the tobacco extracts, described above, and 48 (60%) remained negative. Of the 48 subjects with negative skin test to tobacco, 43 revealed no significant change in measured peripheral blood flow after smoking; the remaining 5 subjects showed a significant decrease in blood flow. On the other hand, of the 32 subjects with positive skin test to tobacco, 21 (65.6%) showed a decrease in blood flow after smoking, while 11 showed no change.

### Discussion

Correlation between skin testing for tobacco extract and blood flow changes after smoking the mixture appears significant as far as negative responses were concerned. It seems from our figures that in a group of "healthy" smokers, simple skin testing might be a fairly reliable way of screening out those subjects in whom smoking will, in all probability, not cause any decrease in peripheral blood flow. This may be expected to comprise about 90% of all negative "skin reactors" in a given group or a total of about 60% of all "healthy" smokers. To screen the remaining 40% for positive blood flow responses would require plethysmographic measurements. The latter procedure is so much more cumbersome and time-consuming than skin testing that it would be of notable practical advantage to first use skin testing if and when screening is desired. The same type of correlative testing is now being applied to healthy non-smokers and to smokers and non-smokers with occlusive arterial diseases and will be reported upon completion.

There remains the fact, that responses in surface temperature and in plethysmographically measured blood flow of the lower extremity did not correlate in this group of 80 healthy subjects. The significance of this has to be ascertained through further work. The clue might well be that blood flow to the leg indicates predominately muscle flow, while surface temperature is a function of skin flow only.

### Summary

Of a group of 80 healthy smokers, 32% showed a decrease in plethysmographically measured extremity flow in response to smoking. Comparison with skin testing revealed a significant correlation in negative responses only; 90% of those who did not react to skin testing with tobacco extract had no decrease in peripheral blood flow in response to smoking.

Committee:  
Dr. Cattell, Chm.  
Dr. Comroe  
Dr. Bing

TOBACCO INDUSTRY RESEARCH COMMITTEE

150 East Forty Second Street, New York 17, N.Y.

#213R1  
Cf. #33  
Activated 2/1/55  
Renewed 5/11/56  
#160  
Activated 8/1/57

Application for Renewal of Research Grant

Date: February 1, 1960

1. Name of Investigator: Marion B. Sulzberger, M.D.  
Walter Redisch, M.D.  
Vincent J. Fontana, M.D.
2. Title: a) Professor and Chairman, Dept. of Dermatology, NYU-Bellevue Medical Center Post-Graduate Medical School.  
b) Associate Professor of Clinical Medicine, NYU College of Medicine.  
c) Assistant Professor of Pediatrics (Allergy Division) NYU College of Medicine.
3. Institution & Address:  
  
New York University-Bellevue Medical Center  
550 First Avenue  
New York 16, New York
4. Project or Subject: Investigation of the effects of tobacco on the human vascular system, based on the fact that certain tobacco effects are due to allergic susceptibility of specific individuals rather than to obligatorily toxic products of tobacco smoke. And that patients with occlusive vascular diseases respond differently than healthy smokers.
5. Detailed Plan of Procedure: The plan outlined in the application of 1959 is to continue. Results to date have been published in part.
  - 1) "Studies in Tobacco Hypersensitivity", J. Allergy, 30:241, 1959 (reprint attached).
  - 2) A paper entitled "Vascular Responses To Smoking Tobacco Compared With Responses to Skin Testing of Tobacco Extracts" - has been accepted for publication in the Annals of Internal Medicine. A copy of the paper is attached.

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- 3) Further results are to be presented at the Conference to be held under the auspices of the New York Academy of Sciences on March 24, 25, and 26. The presentation will be on "Tobacco Hypersensitivity": I. Allergic Implications, II. Peripheral Circulatory Implications. Vincent J. Fontana and Walter Redisch. The presentation will be in essence concerned with 20 subjects in whom detailed correlations between skin sensitivity to specific tobaccos and peripheral blood flow responses were studied. A close correlation was found in this pilot study. It further emanates from differential calculations of skin flow and muscle flow that the decrease in surface temperature and the decrease in skin flow correlate to some degree, while muscle flow most of the time moves in the opposite direction.

At least two more years will be needed to apply the cumbersome technique of separation of skin flow and muscle flow to all groups under study and to arrive at the definite figures representative for the various groups.

6. Budget Plan:

Salaries	\$10,000 per annum
Expendable Supplies	500 per annum
Permanent Equipment	2,000 " "
Overhead (20%)	2,500 " "
Other	
Total	\$15,000

(The total budget to be utilized as specifically stated in application request of 1959)

7. Anticipated Duration of Work: Two years
8. Facilities and Staff Available: Complete facilities and patients and selected staff of the Departments specified above at the New York Skin and Cancer Unit, University Hospital, Bellevue Hospital, Gouverneur Hospital and Goldwater Memorial Hospital. Also other teaching services of the New York University Bellevue Medical Center.
9. Additional Requirements: Supply of specially prepared cigarettes of pure tobacco of different origin and types (Burley, Virginia, Turkish). Also tobacco smoke condensate in form acceptable for skin testing.
10. Additional Information (Including relation of work to other projects and other sources of supply):

Dr. Sulzberger, Dr. Redisch, and Dr. Fontana, are all intimately concerned and familiar with the essential problems in this project, having been

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engaged in this study for at least 3 years. The staff of technical assistants have also been thoroughly trained and are capable of undertaking their assignments in this project.

In the course of work on a project for the NIH, in the laboratories at NYU Research Service, Goldwater Memorial Hospital, a technique has been developed for the separation of skin flow and muscle flow, including a mathematical formula for their calculation. These calculations are now being applied to the experiments concerning vascular responses to tobacco smoking.

/s./ Marion B. Sulzberger, M.D.  
Director of Project

/s./ Edward F. Smith  
Business Officer of the Institution

1003541307

TOBACCO INDUSTRY RESEARCH COMMITTEE  
150 EAST FORTY SECOND STREET NEW YORK 17, N.Y.Application For Research GrantDate: August 9, 1957  
(Resubmitted 4/24/58)

1. Name of Investigator: **F. William Sunderman, M. D., Ph.D.**
2. Title: **Director of the Division of Metabolic Research  
Clinical Professor of Medicine**
3. Institution  
& Address: **Jefferson Medical College  
1025 Walnut Street  
Philadelphia 7, Pa.**
4. Project or Subject: **Metabolism of Trace Metals: Role of Metallic Carbonyls  
in Pulmonary Carcinogenesis**

## 5. Detailed Plan of Procedure (Use reverse side if additional space is needed):

The proposed investigations will be directed toward studying the effects produced by long-continued exposure of experimental animals to repeated sublethal inhalations of metallic vapors. Attention will be focused on the carcinogenicity and the intermediary metabolism of these materials. The chronic effects from inhalation of metallic vapors will be investigated as they may pertain to the inhalation of tobacco smoke,

Background Information

For the past several years our laboratory has been interested in the toxicity of various metallic carbonyls used by industry. (Reprints are attached.)

Metallic carbonyls are formed from the reaction of carbon monoxide with metallic ions, including nickel, cobalt, iron, etc. Nickel carbonyl,  $\text{Ni}(\text{CO})_4$ , for example, is one of the most toxic compounds encountered industrially. Its high volatility makes it difficult to avoid exposure by inhalation during handling. To avoid effects of acute poisoning, the maximal allowable concentration has been set at 0.04 parts per million in air.

Over the past score of years a number of reports have appeared in the literature attributing carcinogenic properties to the inhalation of metallic carbonyls, especially nickel carbonyl. Most of the evidence has

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been obtained from studies on workers in the nickel industry who developed cancer of the respiratory passages after exposure to vapors of nickel carbonyl over a period of ten or more years. The carcinogenic property of nickel carbonyl was first observed by Baader in 1924 and the first report that an excessive number of cases of carcinoma of the lungs and nasal passages developed among nickel workers was made by Grenfell in 1932. An analysis of death certificates issued in South Wales between 1907 and 1934 indicated that 34% of the cases of cancer of the respiratory organs occurred in nickel workers. Barnett noted that, from 1923 to 1948 inclusive, 49 cases of cancer of the nose with 46 fatalities and 82 cases of cancer of the lungs with 72 fatalities were reported from nickel workers in England.

Our laboratory has studied the effects of acute and chronic exposures of nickel carbonyl in rats. Although rats are highly resistant to pulmonary carcinoma, nevertheless, <sup>30 + 4000</sup> squamous metaplasia of the bronchial epithelium has recently been encountered in surviving animals following chronic exposure to nickel carbonyl. Our evidence thus far is suggestive (although inconclusive) that chronic exposure to nickel carbonyl may produce cancer of the respiratory passages in the rat. It is our desire to extend these studies and to correlate them with the effects of inhalation of tobacco smoke.

1003541309

6. Budget Plan:

**Proposed Budget  
for First Year**

Salaries	\$10,800
Expendable Supplies	1,500
Permanent Equipment	3,250
Overhead	1,875
Other (Travel etc.)	250
Total	\$17,675

7. Anticipated Duration of Work: **Four years**

8. Facilities and Staff Available:

Our laboratory is staffed with experienced investigators and technicians and is well equipped for metabolic studies. In addition, our division maintains a toxicity laboratory with special animal quarters. The toxicity laboratory is equipped with a constant flow chamber for exposure of experimental animals to gases and volatile liquids (see reprint for description of chamber).

9. Additional Requirements:

**Staff** - A competent physician-investigator will be available in September for work on this project.

**Equipment** - A spectrophotometer with fluorimeter attachment will be needed. Eventually there will be need for a spectrograph.

10. Additional Information (Including relation of work to other projects and other sources of supply):

This work would extend, compliment and correlate with studies on the toxicity of nickel carbonyl that are being undertaken for the Atomic Energy Commission.

/s./ George A. Bennett, M. D. Dean

Signature /s./ F. William Sunderman  
Director of Project

/s./ George M. Ritchie  
Business Officer of the Institution

George M. Ritchie, Controller

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RESEARCH & DEVELOPMENT

Salaries  
Expensible Supplies  
Equipment  
Overhead  
Other  
Travel etc.

Let with experienced investigators and  
trained for analytical studies. In addition,  
investigator with special animal experience  
will be assigned to conduct the research for  
animal studies and volatile liquids (see  
attached).

Investigator will be available in  
work on this project.  
Investigator with fluorometer attachment will be  
Essentially there will be need for a spectrograph.  
in of work to other projects and other sources of supply).

Equipment and materials with needed on the  
that are being requested for the Atomic Energy

/s/ George A. Bennett, M. D. Dean

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Signature of Project Director

#175

5. Detailed Plan of Procedure (Use reverse side if additional space is needed):

## Background Information

Metallic carbonyls are formed from the reaction of carbon monoxide with metallic ions, including nickel, cobalt, iron, etc. Nickel carbonyl,  $\text{Ni}(\text{CO})_4$ , for example, is one of the most toxic compounds encountered industrially. Its high volatility makes it difficult to avoid exposure by inhalation during handling. To avoid effects of acute poisoning, the maximal allowable concentration has been set at 0.04 parts per million in air.

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been obtained from studies on workers in the nickel industry who developed cancer of the respiratory passages after exposure to vapors of nickel carbonyl over a period of ten or more years. The carcinogenic property of nickel carbonyl was first observed by Reader in 1924 and the first report that an excessive number of cases of carcinoma of the lungs and nasal passages developed among nickel workers was made by Grenfell in 1932. An analysis of death certificates issued in South Wales between 1907 and 1934 indicated that 34% of the cases of cancer of the respiratory organs occurred in nickel workers. Barnett noted that, from 1923 to 1948 inclusive, 49 cases of cancer of the nose with 46 fatalities and 82 cases of cancer of the lungs with 72 fatalities were reported from nickel workers in England.

Our laboratory has studied the effects of acute and chronic exposures of nickel carbonyl in rats. Although rats are highly resistant to pulmonary carcinoma, nevertheless, squamous metaplasia of the bronchial epithelium has recently been encountered in surviving animals following chronic exposure to nickel carbonyl. Our evidence thus far is suggestive (although inconclusive) that chronic exposure to nickel carbonyl may produce cancer of the respiratory passages in the rat. It is our desire to extend these studies and to correlate them with the effects of inhalation of tobacco smoke.

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The proposed investigations will be directed toward studying the effects produced by long-continued exposure of experimental animals to repeated sublethal inhalations of metallic vapors. Attention will be focused on the carcinogenicity and the intermediary metabolism of these materials. The chronic effects from inhalation of metallic vapors will be investigated as they may pertain to the inhalation of tobacco smoke.

#### Background Information

For the past several years our laboratory has been interested in the toxicity of various metallic carbonyls used by industry. (Reprints are attached.)

Metallic carbonyls are formed from the reaction of carbon monoxide with metallic ions, including nickel, cobalt, iron, etc. Nickel carbonyl,  $\text{Ni(CO)}_4$ , for example, is one of the most toxic compounds encountered industrially. Its high volatility makes it difficult to avoid exposure by inhalation during handling. To avoid effects of acute poisoning, the maximal allowable concentration has been set at 0.04 parts per million in air.

Over the past score of years a number of reports have appeared in the literature attributing carcinogenic properties to the inhalation of metallic carbonyls, especially nickel carbonyl. Most of the evidence has



## 6. Budget Plan:

Proposed Budget  
for First Year

Salaries	\$10,800
Expendable Supplies	1,500
Permanent Equipment	3,250
Overhead	1,875
Other (Travel etc.)	250
Total	\$17,675

## 7. Anticipated Duration of Work: Four years

## 8. Facilities and Staff Available:

Our laboratory is staffed with experienced investigators and technicians and is well equipped for metabolic studies. In addition, our division maintains a toxicity laboratory with special animal quarters. The toxicity laboratory is equipped with a constant flow chamber for exposure of experimental animals to gases and volatile liquids (see reprint for description of chamber ).

## 9. Additional Requirements:

Staff - A competent physician-investigator will be available in September for work on this project.

Equipment - A spectrophotometer with fluorimeter attachment will be needed. Eventually there will be need for a spectrograph.

## 10. Additional Information (Including relation of work to other projects and other sources of supply):

This work would extend, compliment and correlate with studies on the toxicity of nickel carbonyl that are being undertaken for the Atomic Energy Commission.

Signature \_\_\_\_\_  
Director of Project

Business Officer of the Institution

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STUDIES IN TOBACCO  
HYPERSENSITIVITY

III. Reactions to Skin Tests and  
Peripheral Vascular Responses

VINCENT J. FONTANA, M.D.  
WALTER REDISCH, M.D.  
ROSE LEE NEMIR, M.D.  
MARJORIE K. SMITH, M.D.  
KURT DECRINIS, M.D.  
and  
MARION B. SULZBERGER, M.D.  
New York, N. Y.

From the Department of Dermatology and  
Syphilology, the Department of Pediatrics  
(Allergy Section), New York University  
Post-Graduate Medical School, and the New  
York University Research Service, Gold-  
water Memorial Hospital, New York Uni-  
versity-Bellevue Medical Center

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(Printed in the U. S. A.)

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## STUDIES IN TOBACCO HYPERSENSITIVITY

### III. REACTIONS TO SKIN TESTS AND PERIPHERAL VASCULAR RESPONSES

VINCENT J. FONTANA, M.D., WALTER REDISCH, M.D., ROSE LEE NEMIR, M.D.,  
MARJORIE K. SMITH, M.D., KURT DECINIS, M.D., AND  
MARION B. SULZBERGER, M.D., NEW YORK, N. Y.

THE problem of tobacco hypersensitivity has been the subject of several controversial reports. The investigations of Harkavy, Hebal, and Silbert<sup>1</sup> and of Sulzberger<sup>2</sup> were the first to indicate the possible allergic activity of tobacco as a cause of certain vascular diseases, especially thromboangiitis obliterans. On the other hand, reports by Trasoff and associates,<sup>3</sup> Chobot,<sup>4</sup> and Westcott and Wright<sup>5</sup> failed to substantiate the conclusion that the skin reaction produced by tobacco extract is a specific response designating a sensitization process of the blood vessels.

The contradictory evidence concerning the role of allergy to tobacco as a cause of vascular disease is succinctly summarized by Lowell in a recent work edited by Wynder.<sup>6</sup> An excellent and complete review on the subject of the immunologic aspects of tobacco and smoking has been published by Silvette, Larson, and Haag.<sup>7</sup> The latter authors include in their summary the statement: "A well designed and properly controlled program of clinical investigation of tobacco sensitivity in relation to disease is greatly to be desired."

The divergent views and findings in this field prompted us to investigate further the possibility that certain tobacco effects are based on a specific allergic susceptibility of particular persons rather than on obligatorily toxic products in tobacco smoke. While the observations of Sulzberger, Harkavy, and others have verified the immunologic specificity of the skin reaction to tobacco, the clinical significance of these reactions is not always obvious. That serious difficulties are encountered in the interpretation of skin tests must be admitted and understood. The factors upon which success depends are the reliability of the extracts used, the method of testing, the evaluation of the reactions in a single person, and their correlation with the patient's clinical history. These precautions have been considered throughout this investigation. The extracts used in this study have been prepared by laboratory workers experienced in the field. Care was taken in the use of these extracts and in the performance and

This work was supported by grants from the Tobacco Industry Research Committee.

Presented at the fourteenth annual meeting of the American Academy of Allergy in Philadelphia, Pennsylvania, Feb. 3-5, 1958.

From the Department of Dermatology and Syphilology, the Department of Pediatrics (Allergy Section), New York University Post-Graduate Medical School, and the New York University Research Service, Goldwater Memorial Hospital, New York University-Bellevue Medical Center.

Received for publication Feb. 10, 1958.

reading of the skin tests in order to eliminate or reduce to a minimum the possibility of nonspecific false positive reactions and other results of experimental errors.

#### MATERIALS AND METHODS

This report includes the skin reactions and historical data on 641 healthy adult volunteers and 294 children tested with tobacco extracts at the Judson Health Center in New York City. The volunteers were skin tested intracutaneously with the five different tobacco extracts. Approximately 0.02 c.c. of the excitant was injected intradermally, and the site was observed for at least twenty minutes to note the result of the test. The skin-test results were interpreted by the standards proposed by Cooke.\*

In order to eliminate questionable or irritant reactions, only the moderately and markedly positive reactions were considered positive for the purposes of this study. Permanent tracings were obtained on all marked positive skin tests. Normal saline was used as a control.

The tobacco extracts were prepared by Mrs. Margaret B. Strauss, Director, Allergy Laboratory, University Hospital, New York City. Burley, Virginia, and Turkish cured tobaccos were procured from a reputable tobacconist who could assure their purity of type for the preparation of these individual tobaccos. Mixed tobacco extracts were composed of the following popular brands of cigarettes in equal parts (including the tobacco and the cigarette paper): Camel, Chesterfield, Lucky Strike, Old Gold, Pall Mall, and Philip Morris. No filter cigarettes were used.

The usual routine in preparing an extract of allergen is to treat the crude mass with an organic solvent, such as acetone, ether, toluene, or Sovasol in order to remove as much oil, fat, and coloring matter as possible before the aqueous extraction. In general, the oil and dye contain no antigenic fraction important to the inhalant type of allergies and their removal produces a superior type of extract because it is clearer and less colored. However, cigarette smoke contains certain volatile esters, aldehydes, ketones, and other organic substances, parts of which are undoubtedly soluble in organic solvents; these organic substances, which might act as allergens, would be removed if the tobacco mass were first defatted by an organic solvent.

Therefore, two types of mixed tobacco extracts were prepared. One was defatted by washing and decanting with toluene before the aqueous extraction, and the other was treated with no organic solvent before the aqueous extraction. The tobacco mass was then extracted in buffered saline (pH 7.0) for two days. After filtration, the extract was dialyzed against buffered saline for forty-eight hours and then concentrated by allowing the extract, in cellophane sausage casing, to hang in front of an electric fan. After Seitz filtration and sterility tests, each extract was standardized on a protein nitrogen basis according to the method of Stull and Cooke. The tobacco extracts used for testing were in 1,000 units phosphotungstic-acid-precipitable nitrogen strength.

\*Original wheal—negative reaction—0.2 to 0.3 cm. wheal. Slight reaction—0.4 to 0.6 cm. wheal. Moderate reaction—1 to 1.5 cm. wheal. Marked reaction—2 plus cm. with pseudopodia.

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Before skin testing, each subject was thoroughly interrogated as to any personal and familial history of allergy and was questioned specifically about peripheral vascular symptoms and pertinent smoking habits. Each subject was carefully questioned as to the following specific points:

*On Allergy*—Personal or familial occurrence of seasonal coryza, bronchial asthma, urticaria, food sensitivity, and drug sensitivity.

*On Peripheral Vascular Disease*—Evidence of numbness of extremities and fainting spells.

*On Symptoms Directly Related in Time to Tobacco Smoking*.—Coughing, dizziness, headaches, effect on appetite, and cold, numbness, and tingling of the extremities.

Eighty healthy adults who had been skin tested with tobacco extract were studied in the New York University Research Service at Goldwater Memorial Hospital. The vascular responses of these persons were tested by automatic recording of surface temperature and by large-limb venous occlusion plethysmograph in a room with constant temperature and humidity under environmental conditions set at 20° and 25° C. with 55 per cent humidity. Measurements were taken before the subjects smoked a cigarette, and at 2-, 6-, and 20-minute intervals following the onset of the smoking procedure. Changes in blood flow greater than 2 c.c. per 100 ml. of tissue per minute and changes in surface temperature of at least 2° C. were considered significant. Ballistocardiograms and electrocardiograms were also obtained on these volunteers, and they were interpreted by the standard criteria. The cardiovascular measurements were recorded before, during, and after the smoking of special cigarettes composed of a mixture of tobaccos from all the above-mentioned commercial cigarettes.

#### RESULTS

*Cutaneous Reactions to Tobacco Extracts*.—Six hundred forty-one healthy adult volunteers, both smokers and nonsmokers, were skin tested with five different tobacco extracts. Table I shows the number and percentage of cutaneous reactions, ranging from negative to marked, to Burley, Virginia, Turkish, mixed (not defatted), and mixed tobaccos in a group of 641 adults. Of the persons

TABLE I. PERCENTAGE OF VOLUNTEERS TESTED BY REACTIONS TO EACH OF FIVE TOBACCO EXTRACTS BY SMOKING HISTORY

EXTRACT	SMOKERS					NONSMOKERS				
	TOTAL	NEGA-TIVE	SLIGHT	MOD-ERATE	MARKED	TOTAL	NEGA-TIVE	SLIGHT	MOD-ERATE	MARKED
Burley	379	71.5	15.3	5.0	8.2	261	73.6	15.7	5.4	5.4
Virginia	378	70.4	14.3	6.1	9.3	261	73.6	14.6	3.8	8.0
Turkish	379	71.0	15.6	4.0	9.5	261	75.5	12.3	3.8	8.4
Mixed	341	68.3	12.6	5.3	13.8	234	73.5	14.5	2.1	9.8
Mixed (de-fatted)	340	70.3	13.5	7.4	8.8	232	73.7	13.8	5.6	6.9
Total*	380	63.2	12.4	6.1	18.4	261	65.5	15.3	5.4	13.8

\*Each person classified by most severe reaction to any of five extracts. No statistical difference between smokers and nonsmokers.

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tested, 18.4 per cent of the smokers showed markedly positive reactions to one or more of the tobacco extracts, as compared with 13.8 per cent of the nonsmokers. The difference between the percentages of smokers and nonsmokers reacting to each extract was not found to be statistically significant. Table I also indicates that there were more reactions to the mixed brand of tobacco extract that was not defatted. The defatting process would remove an appreciable quantity of esters, aldehydes, and other organic compounds ordinarily thought to be volatilized in the smoke of the cigarette. This mixed extract also contains, besides tobacco, unrelated allergens and irritating chemicals. It was noted that in the positively reacting group, some persons reacted to only one of the extracts, some to two, some to three, some to four, and some to all five extracts. By the skin-test criterion, a person who was skin sensitive to tobacco could therefore be sensitive to only one tobacco and the tobacco product of one provenance and not be sensitive to any other type.

No attempt was made to classify the smokers as occasional, moderate, or heavy smokers, as it was found that such classification would be impossible or would more likely be misleading than instructive. Many of the subjects smoked different amounts at different times, some ranging from one or two cigarettes daily during certain periods of their lives to a pack or more at other periods. If it had been possible for us to make an accurate quantitative breakdown of smoking habits, this might have been informative. We do not consider it essential, however, since the incidence of positive skin tests was not significantly greater in the entire group of smokers as compared with entire group of nonsmokers.

Sex and age did not seem to play any role in the incidence of the reactions to tobacco in the various groups tested.

*Relationship of Peripheral Vascular Symptoms to Cutaneous Tobacco Reactions.*—Table II sets forth the percentage of general vascular symptoms in a group of 377 smokers tested with Virginia tobacco extract. It can be seen from these statistics that peripheral vascular symptoms were approximately twice as

TABLE II. PERCENTAGE OF VOLUNTEERS WITH HISTORY OF PERIPHERAL VASCULAR SYMPTOMS AND REACTIONS TO THE VIRGINIA TOBACCO EXTRACT

TOTAL NUMBER	SKIN TEST	NUMBNESS OF EXTREMITIES (PER CENT)	MUSCULAR CRAMPS OF EXTREMITIES (PER CENT)	COLD EXTREMITIES (PER CENT)	TINGLING OF EXTREMITIES (PER CENT)
Smokers (377)	Positive (57)	28	26	30	30
	Negative (320)	14	11	13	15
Nonsmokers (269)	Positive (31)	16	19	16	10
	Negative (229)	14	13	13	11

A significant association was found between smokers with positive skin reactions to tobacco and:

Numbness of the extremities— $\chi^2 = 6.0$ ;  $n = 1$ ;  $P = < 0.02$ .

Cold extremities— $\chi^2 = 8.6$ ;  $n = 1$ ;  $P = < 0.01$ .

Muscular cramps of the extremities— $\chi^2 = 11$ ;  $n = 1$ ;  $P = < 0.01$ .

Tingling of the extremities— $\chi^2 = 6.6$ ;  $n = 1$ ;  $P = < 0.01$ .

The above results tend to show that among the nonsmokers there appeared to be no association of any of the four symptoms with the positive skin tests to tobacco.

common in the smokers with positive skin tests than in the smokers with negative skin reactions. In the nonsmokers, the incidence of peripheral vascular symptoms was about the same, whether the skin-test reaction to tobacco was positive or negative.

*Relationship of the Allergic State to Cutaneous Tobacco Reactions.*—Table III lists the percentage of volunteers with manifestations of personal allergy and the reactions to the Virginia tobacco extract. This table shows the increased incidence of positive skin reactions to tobacco among the allergic persons, both smokers and nonsmokers. Over twice as many persons with positive reactions reported having seasonal rhinitis, bronchial asthma, or urticaria as compared with the subjects with negative skin reactions. In the group of smokers reporting a history of allergic manifestations, 53.4 per cent were found to react in a positive manner to one or more of the tobacco extracts. In contrast, only 18.7 per cent of the normal nonallergic smokers reacted to the tobacco extract. Among the nonsmokers, 40.9 per cent of the allergic persons were found to give positive reactions, and 14.7 per cent of the normal nonsmokers reacted positively to the tobacco extract. This finding would seem to indicate that the reaction to the tobacco extract may be evidence of multiple sensitization characteristic of the allergic state. A skin sensitivity to tobacco could well be more readily acquired by the generally allergic person than by the generally less allergic one on intimate or casual contact with tobacco smoke and tobacco products.

TABLE III. PERCENTAGE OF VOLUNTEERS WITH MANIFESTATIONS OF PERSONAL ALLERGY AND REACTIONS TO THE VIRGINIA TOBACCO EXTRACT

TOTAL NUMBER	SKIN TEST	SEASONAL RHINITIS (PER CENT)	BRONCHIAL ASTHMA (PER CENT)	URTICARIA (PER CENT)	OTHERS* (PER CENT)
Smokers (377)	Positive (57)	7	18	26	37
	Negative (320)	4	4	5	18
Nonsmokers (260)	Positive (31)	19	19	16	13
	Negative (229)	7	4	7	16

\*Food sensitivity, drug sensitivity, and rashes.

Among smokers there was no significant association between positive skin tests to tobacco and seasonal rhinitis.

Among smokers a significant association was found between positive skin tests to tobacco and:

Bronchial asthma— $\chi^2 = 12.9$ ;  $P = < 0.01$ .

Urticaria— $\chi^2 = 26.2$ ;  $P = < 0.01$ .

Among nonsmokers no significant association was noted between the positive skin test reaction to tobacco and the manifestations of personal allergy.

*Symptoms Related to Cigarette Smoking and the Cutaneous Reactions to Virginia Tobacco Extract.*—In Table IV it is noted that 24 per cent of the smokers with positive tests to tobacco gave evidence of coughing spells directly related to the act of smoking, as compared with 12 per cent of the smokers who had negative skin reactions. Symptoms of cold, numbness, and tingling of the extremities associated with smoking were reported by 8 per cent of the smokers with positive tests and by 2 per cent of those with negative reactions. There

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was no apparent difference in incidence of dizziness, headache, and effect on appetite among the smoking group, whether they reacted in a positive or negative manner to the tobacco extract.

TABLE IV. PERCENTAGE OF SMOKERS WITH HISTORY OF TOBACCO SYMPTOMS AND REACTIONS TO THE VIRGINIA TOBACCO EXTRACT

TOTAL NUMBER	SKIN TEST	COUGHING (PER CENT)	DIZZINESS (PER CENT)	HEADACHES (PER CENT)	EFFECT ON APPETITE (PER CENT)	COLD, NUMBNESS, AND TINGLING OF EXTREMITIES (PER CENT)
57	Positive	24	10	6	8	8
320	Negative	12	10	2	6	2

A significant association was found between the positive skin test to tobacco and the incidence of:

Coughing when smoking— $\chi^2 = 5.2$ ;  $P = < 0.02$ .

Cold numbness, and tingling of the extremities— $\chi^2 = 5.5$ ;  $P = < 0.02$ .

*Relationship of Allergy, Peripheral Vascular Symptoms, and Cutaneous Reactions to the Tobacco Extracts.*—Table V is presented in an attempt to correlate allergic manifestations, peripheral vascular symptoms, and the skin reactions to tobacco extract. It was found that 33 per cent of ninety-six smokers with positive skin tests to tobacco gave a history of allergic manifestations and reported having symptoms of peripheral vascular disturbances. Only 10 per cent of 291 smokers with negative skin tests gave evidence of both allergy and peripheral vascular symptoms. Among the nonsmokers, whether they gave positive or negative reactions, no significant difference in incidence of allergy and vascular symptoms was noted.

TABLE V. NUMBER OF SMOKERS WITH PERSONAL ALLERGY AND PERIPHERAL VASCULAR SYMPTOMS AND SKIN REACTIONS TO TOBACCO EXTRACTS

	POSITIVE SKIN TEST	NEGATIVE SKIN TEST	TOTAL NUMBER
Positive allergy, positive peripheral vascular symptoms	32 (33%)	30 (10%)	62
Positive allergy, negative peripheral vascular symptoms	17 (18%)	71 (25%)	88
Negative allergy, positive peripheral vascular symptoms	13 (14%)	35 (12%)	48
Negative allergy, negative peripheral vascular symptoms	34 (35%)	155 (53%)	189
Total number	96	291	387

$\chi^2 = 30.0$ ;  $n = 3$ ;  $P = < 0.001$ .

*Vascular Responses to Smoking and Cutaneous Reactions to Tobacco Extracts.*—Tables VI and VII list the skin temperature and blood flow changes in eighty healthy adults, with their skin-test reactions to tobacco extracts. From this study, it was learned that blood flow and skin temperature changes appear to be independent, since few persons gave reactions to both of these vascular responses. With respect to changes in blood flow, no significant difference was found between those giving positive and those giving negative skin tests to tobacco extracts. On the other hand, skin temperature changes

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were noted in 28 per cent of the smokers giving positive skin-test reactions and in only 4 per cent of those giving negative reactions. The difference is statistically significant.

TABLE VI. CHANGES IN SKIN TEMPERATURE AND BLOOD FLOW ON EIGHTY SMOKERS AND SKIN REACTIONS TO TOBACCO EXTRACTS:

	BURLEY		VIRGINIA		TURKISH		MIXED		MIXED DEFATTED	
	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE
T+ B+	1		1		1		1		1	
T+ B-	6	4	5	5	5	5	4	6	5	5
T- B+	9	11	7	13	7	13	9	11	7	13
T- B-	8	41	9	40	10	39	12	37	8	41
Total	24	56	22	58	23	57	26	54	21	59

T = Skin temperature. + = Positive.  
B = Blood flow. - = Negative.

The electrocardiographic and ballistocardiographic recordings were not included in this discussion, since so few volunteers presented any significant changes and since skin tests with tobacco extracts did not appear to be correlated with the results obtained.

TABLE VII

	POSITIVE REACTORS	NEGATIVE REACTORS
T+ B+	1	0
T+ B-	8	2
T- B+	11	9
T- B-	12	37
Total	32	48

T = Skin temperature. + = Positive.  
B = Blood flow. - = Negative.

Positive reactors to the tobacco extracts are more likely to show changes in skin temperature after smoking:  $\chi^2 = 2.6$ ;  $n = 1$ ;  $P = < 0.01$ .

*Cutaneous Reactions to Tobacco Among Allergic and Nonallergic Children.*—A total of 294 children between the ages of 1 and 5 years were skin tested with the five tobacco extracts. Of this total, 11.5 per cent were found to react in a markedly positive manner to one or more of the tobacco extracts. Thorough questioning of the parents of 269 of the children revealed no evidence of any allergic manifestations. Therefore, these children were considered normal for purposes of this study. Of these normal children, 6 per cent reacted positively to tobacco on skin testing. Twenty-five of the children were found to be allergic and, of these, 64 per cent gave evidence of skin sensitivity to the tobacco extract on skin testing. None of these children had ever smoked, but they had had the same exposure to tobacco smoke and other tobacco products as other nonsmokers. Practically all of these children were exposed to one or more smokers within the immediate family at home. Here again, as in the adult group, it would seem that the generally allergic disposition of the person renders him very receptive to the development of multiple skin sensitization, including

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sensitization to tobacco, on casual or intimate exposure to the allergen. There was no difference in the incidence of positive skin reactions to tobacco in the various age groups. Female subjects reacted in the same proportions as male subjects.

#### SUMMARY AND INFERENCES

A group of 641 healthy volunteers were questioned concerning personal allergies, smoking history, symptoms presumed to be connected with smoking, and peripheral vascular disturbances.

These persons were skin tested by the intradermal method with five different tobacco extracts: Burley, Virginia, Turkish, and mixed tobaccos. The mixed tobacco extract was made by combining tobaccos from six popular cigarettes.

It was found that about 15 per cent of 641 adults reacted in a positive manner to one or more of the tobacco extracts. The per cent reacting was essentially the same in the smokers as in the nonsmokers.

It might have been argued that the heavier smokers, by exposing themselves to more tobacco smoke, became skin reactive to tobacco extracts more frequently. This argument scarcely appears valid, however, in view of our finding that smokers did not react to skin tests with tobacco extracts significantly more often than nonsmokers.

An increased incidence of peripheral vascular symptoms was noted in the smokers with positive reactions to the tobacco, as compared with the smokers who did not react on skin testing with the tobacco.

It might also be argued that excessive smoking could lead to increased incidence of skin-test reactions to tobacco and that the amount of smoking might be an indication of the degree of the patient's anxiety and tension.

In view of the absence of any difference between smokers and nonsmokers in incidence of skin reactions to tobacco, however, it appears extremely unlikely that the degree of anxiety and tension could be a factor in producing skin-test reactions. Moreover, in similar fashion, since the increased smoking could not be proved responsible for increased skin reactions to tobacco extracts, increased anxiety and nervousness could not explain the increased incidence of peripheral vascular symptoms in the smokers with positive skin tests to tobacco as compared to those with negative skin tests.

Volunteers with positive tests to the tobacco extracts reported a history of nontobacco-related allergic manifestations more frequently than the group with negative reactions.

Of the smokers with positive skin reactions to tobacco extracts 33 per cent gave a personal history of allergic manifestations and peripheral vascular symptoms, as compared with 10 per cent of the smokers who did not react to the skin tests with tobacco extracts.

Eighty of these persons were investigated further for circulatory disturbances by means of plethysmographic measurements of blood flow to the lower extremities and automatic recording of surface temperature. These

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tests were performed before, during, and after the smoking of a mixed-tobacco cigarette. These experiments indicated that, on smoking the mixed-tobacco cigarette, 23 per cent of the smokers with positive skin tests to tobacco had changes in peripheral circulation indicated by skin temperature as compared with the smokers with negative skin tests, of whom 4 per cent had changes in skin temperature.

In a group of 294 children between the ages of 1 and 5 years tested with the tobacco extracts, 11.5 per cent reacted in a positive manner to one or more of the tobacco extracts. In the small group of allergic children tested, 64 per cent gave evidence of skin sensitivity to the tobacco extracts as compared with 6 per cent of a nonallergic control group.

The close and consistent parallelism between the positive cutaneous reactions to tobacco extract and symptoms of peripheral vascular disturbances, tobacco symptoms, and personal history of allergic manifestations might possibly serve as a clue to the interpretation of the tobacco reactions that we obtained. The findings indicate that our positive skin-test reactions to tobacco extracts were based on an allergic (that is, immunologic) mechanism and were associated in more than normal incidence with hypersensitivity to other allergens as well as with fall of peripheral temperature on smoking and with certain clinical peripheral vascular symptoms. It is felt that, although the number of volunteers tested is small, there is a suggestion that the skin test with tobacco may be helpful as a "screening" test to aid in determining the possible importance of tobacco as an etiological factor in certain forms of peripheral vascular symptomatology.

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